REMARKS

Claims 1, 46, and 48-50 are pending in the application. Claims 1 and 46 through 50 are rejected.

In the Claims

Claim 1 was amended to correct a typographical error. The amendment is supported by the as-filed application and no new matter has been added.

35 U.S.C. § 112 Claim Rejections

As set forth on page 2 of the outstanding Office Action Claims 1 and 46-50 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to adequately teach how to use the instant invention. More specifically, it is alleged that the reasons for this rejection are of record on page three of the Office Action mailed 12/6/04. On page three of the Office Action mailed 12/6/04, it was alleged that "since the claimed invention is not supported by a specific substantial credible asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention." Thus, the enablement rejection is solely based on the § 101 rejection. Since Applicants have disclosed a specific, substantial and credible utility, as indicated by the Examiner's withdrawal of the utility rejection, this rejection is rendered moot. Applicants therefore respectfully request withdrawal of this rejection.

A new § 112 enablement rejection is also given here alleging that the breadth of claims is excessive in view of the 90% homology limitation. In particular, it is alleged that Applicants provide no guidance or working examples of proteins which are at least 90% identical to the full length protein and it is not predictable to one of ordinary skill in the art how to make a functional protein which is less than 100% identical.

Applicants note that the biochemical identities of each of the claimed proteins were known at the time of filing. AKT1, AKT2, FNTA, TPRD, KIAA0728, PPL, Golgin-84, CL1C1, and ARK7A2 all had defined amino acids sequences and structures at the time of filing. Just by way of example, a number of pre-filing date references are submitted herewith illustrating the great amount of knowledge regarding AKT structure

and function of various AKT homologs having less than 100% homology to AKT. Hsu *et al.* (*Blood* 98:2853 (1991)) disclose a dominant negative kinase inactive mutant akt (K179M). Kitamura *et al. Mol. Cell Biol.* 18:3708 (1998) discloses dominant negative mutants of AKT where the threonine and serine phosphorylation sites on AKT (threonine 308 and serine 473) implicated in activation were mutated to alanine (see also Kohn *et al. J. Biol. Chem.* 273:11937-11943 (1998)).

It is noted that no objective evidence has been presented by the Patent Office to question Applicants presumption of enablement. The evidence of record indicates that the skilled artisan can make and use the invention commensurate with the scope of the claims. Since the Patent Office has not provided any evidence to challenge Applicants presumptively correct assertion of enablement, Applicants respectfully request withdrawal of this rejection.

As set forth on Page 4 of the Office Action, claims 1, 46, and 48 through 50 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject which was not described in the specification in such a way to reasonably covey to one skilled in the art that the inventor had possession of the claimed invention. It is alleged that the specification and claims do not provide any guidance as to what changes should be made and no common structural attributes identify the members of the genus. Applicants respectfully traverse this rejection.

It is first noted that the Patent Office bears the initial burden of establishing that Applicant's specification does not satisfy the written description requirement under 35 U.S.C. 112, first paragraph. See In re Wertheim, 541 F.2d 257, 191 USPQ 90 (CCPA 1976). The claims under examination are drawn to methods of screening protein-protein interactions. To meet the burden, the Office Action must establish with a preponderance of evidence that a person skilled in the art would not recognize that the inventor had possession of the claimed protein complexes. Applicants respectfully submit that the Patent Office has clearly not met this burden.

"The claimed subject matter need not be described in haec verba to satisfy the description requirement. It is not necessary that the application describe the claim limitations exactly, but only so clearly that one having ordinary skill in the pertinent art

would recognize from the disclosure that appellants invented processes including those limitations." In re Herschler, 591 F.2d 693, 700, 200 USPQ 711, 717 (CCPA 1979). According to the Federal Circuit, "the written description requirement can be met by 'showing that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics ... i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.'" Enzo Biochem, Inc. v. Gen-Probe Inc., 296 F.3d 1316, 1324, 63 USPQ2d 1609, 1613 (Fed. Cir. 2002).

Consistent with the Federal Circuit, the PTO's Revised Interim Written Description Guidelines Training Materials (hereinafter "Training Materials") is also instructive. For example, in Example 14 of the Training Materials, the specification discloses a single species of the claimed protein genus. "The specification also contemplates but does not exemplify variants of the protein wherein the variant can have any or all of the following: substitutions, deletions, insertions and additions. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and provides an assay for detecting the catalytic activity of the protein." USPTO Revised Interim Written Description Guidelines Training Materials, page 53 (emphasis added). The Training Material holds that the claim in Example 14, which defines a protein variant by (1) sequence identity and (2) its activity (catalytic activity), meets the written description requirement.

In the instant application under examination, the specification clearly describes that the specified proteins interact. The chemical structures of such interacting fragments are clear in view of the GenBank references in Table 11 at page 23 of the specification and the fact that these proteins are known in the art. As a skilled artisan would recognize, the interaction between the claimed proteins (having the amino acids coordinates in Table 11, page 23), is determined by the sequences of such proteins and protein fragments. Clearly, sufficiently detailed structural characteristics (the amino acid sequences) and the correlating functional characteristics (binding activities) are disclosed in Applicant's specification consistent with Enzo, and the Training Material.

Applicants note that specification provides considerable guidance regarding identifying common structural elements and attributes that delineate the genus to the skilled artisan at the time the application was filed. In particular, binding fragments, representative of interacting domains of the claimed protein complexes, are disclosed in Table 11 on page 23 of the specification. The meaning of 90% homology is defined in paragraph 57 of the specification. Methods for determining whether a homologue interacts with another protein are disclosed in paragraphs 31-41 and Example 1 (paragraphs 63-64). Paragraphs 19-28 illustrate that the functions and structures of the proteins claimed herein are known with known attributes and characteristics. The homologues, they are defined by (1) percentage identity and (2) binding activity, consistent with Example 14 of the Training Material.

Further, the specification also provides detailed teachings on how to determine or detect the interaction between two polypeptides (e.g., full proteins, protein fragments, homologues, and fusion proteins). In other words, the Specification describes assays for determining the activity of the claimed proteins (i.e. the binding affinity to the interacting partner). See e.g., Examples 14 and 15 at paragraphs 0074-0078. Indeed, such and other assays for protein-protein interactions are routine and well-known in the art. These assays are much more routine and predictable than the enzymatic assays in Example 14 of the Training Materials, if comparable at all.

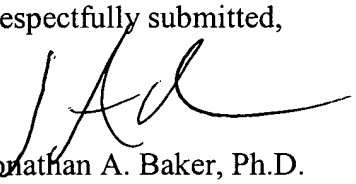
It is submitted that such descriptions in the specification constitutes sufficiently detailed, relevant identifying characteristics of the claimed subject matter consistent with Enzo. The Office Action has failed to establish why one of ordinary skill in the art, provided with the descriptions of the specification discussed above, would be unable to recognize that the inventor invented and has in possession, the method of using the claimed protein complexes and methods of use. Applicants therefore request withdrawal of the rejection.

Since the skilled artisan recognizes proteins having 90% identity to the claimed proteins and can readily identify proteins that have the recited binding characteristics using a well-known method that is described in the specification, Applicants request that the § 112, first paragraph, rejection be withdrawn.

CONCLUSION

Claims 1, 46, and 48-50 are believed to be in condition for allowance, and an early notice thereof is respectfully solicited. Should the Examiner determine that additional issues remain which might be resolved by a telephone conference, he is respectfully invited to contact Applicants' undersigned attorney. Since the last Office Action is final, Applicants are concurrently filing a Notice of Appeal herewith.

Respectfully submitted,



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Requirement for Activation of the Serine-Threonine Kinase Akt (Protein Kinase B) in Insulin Stimulation of Protein Synthesis but Not of Glucose Transport

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A wide variety of biological activities including the major metabolic actions of insulin is regulated by phosphatidylinositol (PI) 3-kinase. However, the downstream effectors of the various signaling pathways that emanate from PI 3-kinase remain unclear. Akt (protein kinase B), a serine-threonine kinase with a pleckstrin homology domain, is thought to be one such downstream effector. A mutant Akt (Akt-AA) in which the phosphorylation sites (Thr^{308} and Ser^{473}) targeted by growth factors are replaced by alanine has now been shown to lack protein kinase activity and, when overexpressed in CHO cells or 3T3-L1 adipocytes with the use of an adenovirus vector, to inhibit insulin-induced activation of endogenous Akt. Akt-AA thus acts in a dominant negative manner in intact cells. Insulin-stimulated protein synthesis, which is sensitive to wortmannin, a pharmacological inhibitor of PI 3-kinase, was abolished by overexpression of Akt-AA without an effect on amino acid transport into the cells, suggesting that Akt is required for insulin-stimulated protein synthesis. Insulin activation of p70 S6 kinase was inhibited by ~75% in CHO cells and ~30% in 3T3-L1 adipocytes, whereas insulin-induced activation of endogenous Akt was inhibited by 80 to 95%, by expression of Akt-AA. Thus, Akt activity appears to be required, at least in part, for insulin stimulation of p70 S6 kinase. However, insulin-stimulated glucose uptake in both CHO cells and 3T3-L1 adipocytes was not affected by overexpression of Akt-AA, suggesting that Akt is not required for this effect of insulin. These data indicate that Akt acts as a downstream effector in some, but not all, of the signaling pathways downstream of PI 3-kinase.

Akt is a pleckstrin homology (PH) domain-containing protein serine-threonine kinase whose kinase domain shares structural similarity with protein kinase C (PKC) isozymes and cyclic AMP-dependent protein kinase (PKA) (3). Thus, Akt has also been termed RAC-PK (protein kinase related to A and C kinases) (19) and PKB (protein kinase B) (7). Insulin and various other growth factors activate Akt, and this activation is inhibited by pharmacological blockers of phosphatidylinositol (PI) 3-kinase or by a dominant negative mutant of PI 3-kinase (4, 14, 25). Furthermore, Akt is activated by overexpression of a constitutively active mutant of PI 3-kinase in quiescent cells (11, 23). These observations indicate that Akt is a downstream effector of PI 3-kinase.

PI 3-kinase, which consists of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (5), is implicated in various metabolic effects of insulin (18, 59). A dominant negative mutant of PI 3-kinase as well as various pharmacological inhibitors, such as wortmannin and LY294002, have been used to block specific signaling pathways that include this enzyme (6, 16, 31, 39, 61). The metabolic actions of insulin that are sensitive to either a dominant negative mutant or pharmacological inhibitors of PI 3-kinase include stimulation of glucose uptake, antilipolysis, activation of fatty acid synthase and glycogen synthase, and stimulation of amino acid transport and protein synthesis (6, 16, 34, 37, 47, 48, 54, 55). Moreover, regulation of the amounts of specific protein participants in metabolic pathways by insulin is mediated by this lipid kinase (52). Although these observations indicate that PI 3-kinase is a major regulator of the metabolic effects of insulin, the roles of the various downstream effectors of PI 3-kinase in each of these actions remain unclear.

The kinase activity of Akt fused with a viral Gag protein or tagged with a myristoylation signal sequence is higher than that of wild-type Akt (Akt-WT) (4, 26). Overexpression of these mutant Akt proteins induced activation of p70 S6 kinase (4, 26), which is also activated by insulin in a wortmannin-sensitive manner (42, 43). Expression of these active Akt mutants also promoted glucose uptake and translocation of GLUT4 glucose transporters in quiescent adipocytes (27, 53). These observations have suggested that Akt is a downstream effector of PI 3-kinase that mediates insulin-induced activation of p70 S6 kinase and glucose uptake. This proposal could be tested further by investigating the effects of specific inhibition of Akt activity on these actions of insulin. However, a mutant Akt that exerts dominant negative effects has not previously been described.

The mechanism by which Akt is activated in response to growth factor stimulation is not fully understood. PI 3,4-bisphosphate, one of the products of PI 3-kinase action, stimulates Akt activity in vitro (15, 24). Furthermore, Akt mutants with substitutions in or lacking the PH domain were not activated by this phospholipid (15, 24), suggesting that Akt is activated as a result of direct interaction of its PH domain with the lipid. On the other hand, other studies have suggested the importance of phosphorylation of Akt on serine and threonine residues in regulation of its activity. Akt is phosphorylated in vivo in response to various growth factors that stimulate Akt

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activity (4, 14, 26), and dephosphorylation of in vivo-activated Akt by a serine-threonine phosphatase abolished its enzymatic activity (26). Akt is phosphorylated on Thr^{308} and Ser^{473} in response to insulin in vivo, and Akt mutants in which either Thr^{308} or Ser^{473} was substituted were not activated (1). Moreover, a protein kinase which phosphorylates and activates Akt has been cloned and characterized (2, 44, 50, 51). These data have suggested that Akt is primarily activated as a result of its phosphorylation on serine and threonine residues by an upstream kinase.

We have now shown that when overexpressed in CHO cells or 3T3-L1 adipocytes, a mutant Akt in which growth factor-targeted serine and threonine phosphorylation sites are replaced with alanine exerted a dominant negative effect on endogenous Akt activity stimulated by insulin. With the use of this dominant negative Akt, we have investigated the roles of Akt in insulin-stimulated protein synthesis, p70 S6 kinase activation, and glucose transport in these cells.

MATERIALS AND METHODS

Cells, plasmids, and antibodies. CHO cells were routinely maintained and 3T3-L1 preadipocytes were maintained and induced to differentiate into adipocytes as described previously (48). To establish CHO cells that stably express FLAG epitope-tagged Akt (CHO-Akt cells), we transfected CHO cells with pSV40-hgh, which confers resistance to hygromycin, and a PECE vector encoding FLAG epitope-tagged rat Akt1 (RAC-PKα) (30). Transfected cells were selected and cloned as described previously (22). PECE vectors encoding FLAG epitope-tagged rat Akt2 (RAC-PKβ) (30) and RAC-PKγ (29) were as described previously. Monoclonal antibodies to the hemagglutinin (HA) epitope tag (12CA5) or to the FLAG epitope tag were obtained from Boehringer Mannheim and Kodak Scientific Imaging Systems, respectively. Polyclonal antibodies to Akt were generated against a glutathione *S*-transferase fusion protein containing amino acids 428 to 480 of rat Akt1. Polyclonal antibodies to mitogen-activated protein (MAP) kinase (αC92) were as described previously (48). Polyclonal antibodies to p70 S6 kinase were generated against a synthetic peptide corresponding to amino acids 2 to 23 of the rat enzyme (33).

Construction of adenovirus vectors. Adenovirus vectors encoding a dominant negative PI 3-kinase (AxCAΔp85) or a dominant negative SOS (AxCAΔSOS) were as described previously (48). Rat Akt1 was tagged with the HA epitope by PCR with a sense primer (5′-ACT AAG CTT GCC ATG TAC CCA TAC GAT GTT CCG GAT TAC GCT AAC GAC GTA GCC ATT GTG AAG G), an antisense primer (5′-GAT GAA TTC ACT GGG TGA ACC TGA CCG G), and a full-length rat Akt1 cDNA as template. Both Thr^{308} and Ser^{473} of HA-tagged rat Akt1 were replaced by alanine with the use of a Quick Change site-directed mutagenesis kit (Stratagene). Lys^{179} of HA-tagged rat Akt1 was replaced with asparate by the use of PCR. These mutants were termed Akt-AA and AktK179D, respectively. DNA encoding the HA-tagged wild-type and mutant (Akt-AA or AktK179D) Akt proteins was subcloned into pAxCAwt (36), and adenovirus vectors containing these cDNAs were generated by transfecting 293 cells with the corresponding pAxCAwt plasmid together with a DNA-terminal protein complex (36), as described previously (48). The resulting vectors were termed AxCAAkt-WT, AxCAAkt-AA, and AxCAAkt-K179D, respectively. CHO cells or 3T3-L1 adipocytes were infected with adenovirus vectors at the indicated multiplicity of infection (MOI) as described previously (48, 57). The cells were subjected to experiments 48 h after infection.

Kinase assays. CHO cells or 3T3-L1 adipocytes were deprived of serum for 16 to 20 h, incubated in the absence or presence of insulin, and then immediately frozen with liquid nitrogen. The MAP kinase assay was performed with immunoprecipitates prepared with antibodies to MAP kinase as described previously (47, 48).

For p70 S6 kinase assays, the frozen cells were lysed in a solution containing 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 1 mM benzamidine, 1 mM EDTA, 6 mM EGTA, 15 mM sodium pyrophosphate, 1% Nonidet P-40, 30 mM *p*-nitrophenyl phosphate, 0.5 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged (at 15,000 × *g* for 20 min), and the resulting supernatant was subjected to immunoprecipitation with polyclonal antibodies to p70 S6 kinase. After being washed three times with HEPES-buffered saline (pH 7.5) containing 0.1% Triton X-100, the immunoprecipitates were incubated for 30 min at 25°C in a reaction mixture (30 μl) containing 50 mM morpholine propanesulfonic acid (pH 7.2), 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM *p*-nitrophenyl phosphate, 100 μM unlabeled ATP, 3.0 μCi of [γ-^{32}P]ATP, and 100 μM S6 synthetic peptide (KRRRLSSLRASTSKSESSQK) as the substrate. The reaction mixture was then centrifuged (at 15,000 × *g* for 3 min), and the resulting supernatant was transferred to P81 (Whatman) filter paper. After extensive washing of the filters with 0.5% phosphoric acid, ^{32}P incorporation into the peptide was determined by liquid scintillation spectroscopy.

To assay the activities of FLAG- or HA-tagged Akt kinase, we lysed the cells in a solution containing 50 mM HEPES-NaOH (pH 7.6), 150 mM NaCl, 1% Triton X-100, bacitracin (1 mg/ml), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, and 30 mM sodium pyrophosphate. The lysates were subjected to immunoprecipitation with antibodies to HA or to FLAG. After being washed three times with HEPES-buffered saline (pH 7.5) containing 0.1% Triton X-100, the immunoprecipitates were incubated for 30 min at 30°C with 3.0 μCi of [γ-^{32}P]ATP in a reaction mixture (30 μl) containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 25 μM unlabeled ATP, 1 μM protein kinase inhibitor, and histone 2B (0.2 mg/ml) as the substrate. The reaction was terminated by the addition of sodium dodecyl sulfate (SDS) sample buffer, and the samples were then fractionated by SDS-polyacrylamide gel electrophoresis on a 15% gel. The radioactivity incorporated into histone 2B was determined with a Fuji BAS 2000 image analyzer.

For assay of endogenous Akt activity in CHO cells or 3T3-L1 adipocytes that had been infected with AxCAAkt-AA, the cells were lysed as described for determination of the activity of epitope-tagged Akt. The lysates were subjected to three sequential immunoprecipitations for 90 min at 4°C (in a final volume of 400 μl) with 20 μl of protein G-Sepharose (Pharmacia) that had been coupled with 20 μg of antibodies to HA. The final supernatant was then subjected to immunoprecipitation with polyclonal antibodies to Akt, and Akt kinase assays were performed with the resulting immunoprecipitates as described above.

Glucose uptake and translocation of GLUT4. Glucose uptake was assayed as described previously (16, 48). In brief, CHO cells and 3T3-L1 adipocytes cultured in six-well plates were incubated for 16 h in Ham's F12 and Dulbecco's modified Eagle's media, respectively, containing 5.6 mM glucose and 0.5% fetal bovine serum. The cells were washed twice with DB buffer (140 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 [pH 7.4], 0.5 mM $MgCl_2$) and incubated with 100 nM insulin for 15 min, after which 1 ml of DB buffer containing bovine serum albumin (BSA; 1 mg/ml) and 0.1 mM 2-deoxy-D-[1,2-^{3}H]glucose (1 μCi) was added to each well. After 5 min, the cells were washed and then solubilized with 0.1% SDS. The radioactivity incorporated into the cells was measured by liquid scintillation spectroscopy.

Translocation of GLUT4 to the plasma membrane was measured by the plasma membrane lawn assay as previously described (48).

Protein synthesis and amino acid transport. Insulin-stimulated protein synthesis was assayed essentially as described previously (34), with the following modifications. CHO cells or 3T3-L1 adipocytes were deprived of serum for 24 h and then incubated for 1 h either with a mixture of Ham's F12 and DB buffer (1:100 [vol/vol]) or with methionine- and cysteine-free Dulbecco's modified Eagle's medium (Sigma), respectively. After addition of Tran^{35}S-label (16 μCi/ml; ICN) and 100 nM insulin, the cells were incubated for an additional 1 h and the medium was then aspirated. The cells were washed three times with phosphate-buffered saline containing 10 mM methionine and then lysed in a solution containing 30 mM Tris-HCl (pH 7.5), 140 mM NaCl, and 0.5% Nonidet P-40. Proteins were precipitated by the addition of ice-cold trichloroacetic acid (final concentration, 10% [wt/vol]) containing 10 mM methionine. The protein precipitates were washed three times with ice-cold phosphate-buffered saline containing 10% trichloroacetic acid and 10 mM methionine and then solubilized in 1 M NaOH at 37°C for 10 min. The protein-associated radioactivity was assayed by liquid scintillation spectroscopy.

Amino acid uptake was assayed as described previously (54), with the following modifications. CHO cells cultured in six-well plates were deprived of serum for 16 h, washed twice with DB buffer, and incubated for 20 min with 2 ml of DB buffer containing BSA (1 mg/ml) and 10 mM unlabeled α-methylaminoisobutyrate (MeAIB). One milliliter of DB buffer containing BSA (1 mg/ml), 10 mM unlabeled MeAIB, 10 μM [^{14}C]MeAIB (1 μCi/ml), and 100 nM insulin was then added to each well; after 30 min, the cells were washed three times with ice-cold DB buffer containing BSA (1 mg/ml) and solubilized with 0.1% SDS. The amount of radioactivity incorporated into the cells was measured by liquid scintillation spectroscopy.

Apoptosis assay. Apoptosis was assayed by measuring characteristic DNA laddering. DNA laddering was analyzed essentially as described previously (20), with the following modifications. CHO cells cultured in 6-cm-diameter plates were infected with AxCAAkt-AA or AxCAAkt-WT at the indicated MOI (PFU/cell). After 32 h, the infected cells were deprived of serum for 16 h, washed once in phosphate-buffered saline, and lysed in 0.1 ml of a buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.5), and 10 mM EDTA. The lysates were incubated for 20 min at 4°C and then centrifuged at 15,000 × *g* for 20 min. The DNA-containing soluble fraction was extracted from the resultant supernatants with phenol-chloroform, ethanol precipitated, resuspended in a buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 20 μg of RNase A per ml, and then incubated at 37°C for 1 h. DNA was loaded onto a 1.5% agarose gel; after electrophoresis, the gel was stained with ethidium bromide and photographed.

RESULTS

Dominant negative effects of a mutant Akt with alanine substitutions at Thr^{308} and Ser^{473}. A rat Akt1 (RAC-PKα) (28) mutant (termed Akt-AA) in which Thr^{308} and Ser^{473} were replaced by alanine and rat Akt-WT were tagged with the HA

epitope at their NH_2 termini and expressed in CHO cells with the use of adenovirus vectors (AxCAAkt-WT or AxCAAkt-AA) containing the corresponding cDNA. The infected cells were then incubated in the absence or presence of insulin, after which recombinant Akt was immunoprecipitated with antibodies to HA and assayed for Akt kinase activity with histone 2B as the substrate. Consistent with previous observations (1), insulin increased the activity of Akt1-WT about 20-fold but had no effect on the activity of Akt-AA (Fig. 1A to C).

Because Akt has been shown to be involved in cell survival signals (13, 20), we first examined the effects of Akt-AA on apoptosis. CHO cells were infected with AxCAAkt-AA or AxCAAkt-WT at the indicated MOI, and then DNA laddering was examined. Infection of CHO cells with AxCAAkt-AA led to DNA laddering in an MOI-dependent manner whereas AxCAAkt-WT did not exhibit such an effect (Fig. 1D), suggesting that the induction of apoptosis was due not to a nonspecific effect of viral infection but to the effect of Akt-AA. However, DNA laddering was not evident with the cells infected with AxCAAkt-AA at an MOI of 20. We thus examined the effects of this mutant on various insulin-induced biological activities in CHO cells infected with AxCAAkt-AA at an MOI of 20 or less.

We also established CHO-Akt cells, which stably express FLAG epitope-tagged rat Akt1. The extent of expression of Akt protein in these cells is approximately seven times that in parental CHO cells (data not shown). CHO-Akt cells were infected with various adenovirus vectors, incubated in the absence or presence of insulin, lysed, and subjected to immunoprecipitation with antibodies to FLAG. Assay of the resulting immunoprecipitates for Akt activity revealed that insulin induced a 6- to 10-fold increase in the activity of FLAG-tagged Akt (Fig. 2 and 3). Infection of the cells with AxCAΔp85 (48), an adenovirus encoding a dominant negative mutant of PI 3-kinase, inhibited insulin-induced activation of Akt (Fig. 2A). In contrast, infection of CHO-Akt cells with an adenovirus encoding a dominant negative mutant of SOS (AxCAΔSOS) (48, 57), even at a virus concentration sufficient for almost complete inhibition of insulin-induced activation of MAP kinase activity in CHO cells (data not shown), had no effect on Akt activity (Fig. 2B). Infection of the cells with AxCAAkt-AA resulted in a dose-dependent inhibition of Akt activity precipitated with antibodies to FLAG (Fig. 3B and C); the extent of inhibition paralleled the expression of Akt-AA protein (Fig. 3A), with ~75% inhibition apparent at an MOI of 20 PFU/cell. We also constructed an adenovirus vector that encodes a mutant Akt in which Lys^{179} in the kinase domain was replaced by asparate (Akt-K179D). This mutant Akt did not exhibit kinase activity (data not shown). Infection of the cells with AxCAAkt-K179D had little effect on Akt activity precipitated with antibodies to FLAG (Fig. 3B and C), whereas the extent of expression of Akt-K179D protein assessed by immunoblot analysis with antibodies to HA was similar to that of Akt-AA protein in the cells infected with AxCAAkt-AA (Fig. 3A).

We next investigated the effect of Akt-AA on endogenous Akt activity in CHO cells by precipitating the endogenous protein with polyclonal antibodies to Akt. The polyclonal antibodies recognize all three known rat Akt isoforms, Akt1 (RAC-PKα), Akt2 (RAC-PKβ), and RAC-PKγ, which were transiently expressed in COS cells (Fig. 4). Because these antibodies recognize both endogenous and recombinant Akt proteins, Akt-AA was immunodepleted from cell lysates with antibodies to HA before endogenous Akt was immunoprecipitated with the polyclonal antibodies and assayed for kinase activity toward histone 2B. Infection of CHO cells with AxCAAkt-AA resulted in the expression of Akt-AA, the electrophoretic mobility of which was slightly less than that of endogenous Akt because of the presence of the epitope tag, in an MOI-dependent manner (Fig. 5A). At an MOI of 20 PFU/cell, the abundance of Akt-AA was ~30 to 50 times that of endogenous Akt (data not shown). After three sequential immuno-

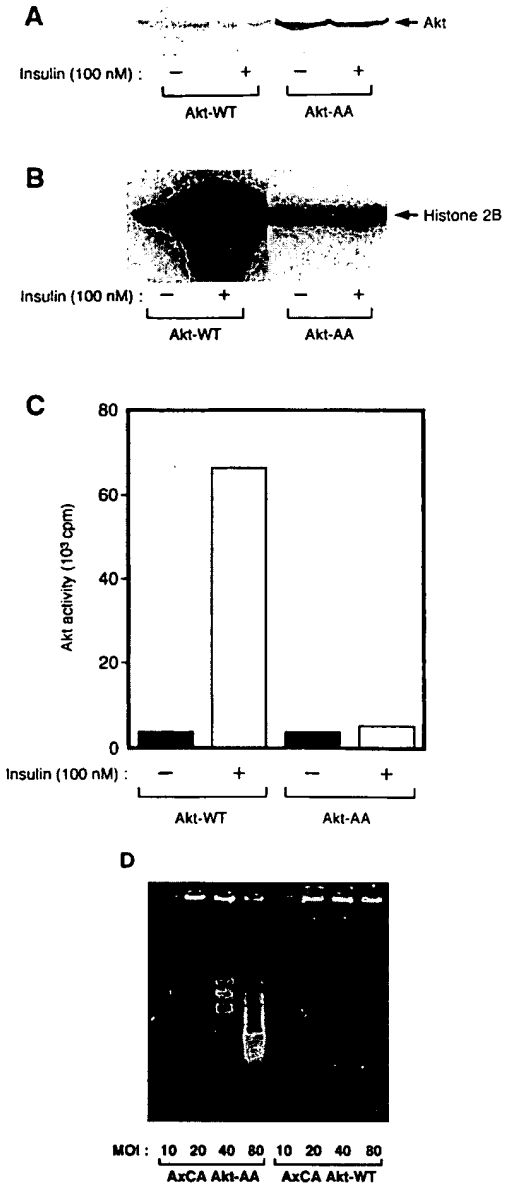

FIG. 1. (A to C) Effects of insulin on the kinase activity of Akt-WT and Akt-AA in CHO cells. CHO cells were infected with either AxCAAkt-WT or AxCAAkt-AA at an MOI of 5 PFU/cell. The cells were incubated for 10 min in the absence or presence of 100 nM insulin, lysed, and subjected either to immunoblot analysis with antibodies to HA (A) or to immunoprecipitation with antibodies to HA (B and C). The immunoprecipitates were assayed for Akt kinase activity, and the radioactivity incorporated into histone 2B was visualized (B) or quantitated (C) with an image analyzer as described in Materials and Methods. Data are representative of three independent experiments. (D) Effects of Akt-AA and Akt-WT on apoptosis in CHO cells. CHO cells were infected with AxCAAkt-AA (left) or AxCAAkt-WT (right) at the indicated MOI (PFU/cell). The cells were deprived of serum for 16 h, and DNA laddering was examined as described in Materials and Methods. Data are representative of two independent experiments.

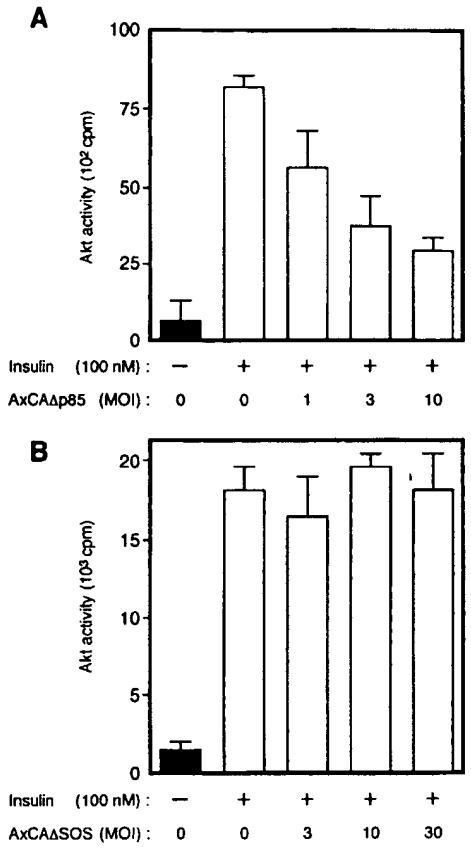

FIG. 2. Effects of Δp85 (A) and ΔSOS (B) on insulin-induced activation of Akt in CHO-Akt cells. CHO-Akt cells were infected with adenoviruses encoding Δp85 (AxCAΔp85) (A) or ΔSOS (AxCAΔSOS) (B) at the indicated MOI (PFU/cell). The cells were incubated for 10 min in the absence or presence of 100 nM insulin, after which lysates from individual 6-cm-diameter plates were subjected to immunoprecipitation with antibodies to FLAG and the precipitates were assayed for Akt kinase activity. Data are means ± standard errors from three experiments.

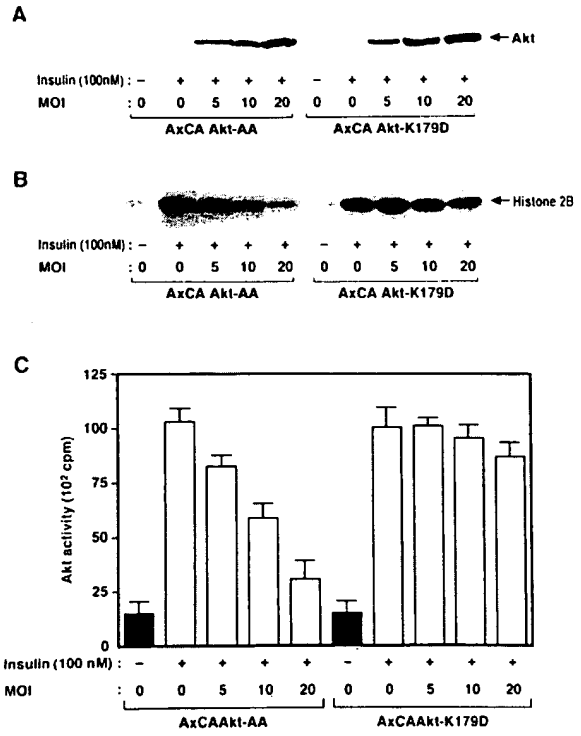

FIG. 3. Effects of Akt-AA and Akt-K179D on insulin-induced activation of Akt in CHO-Akt cells. CHO-Akt cells were infected with an adenovirus encoding Akt-AA (AxCAAkt-AA) or Akt-K179D (AxCAAkt-K179D) at the indicated MOI (PFU/cell), incubated for 10 min in the absence or presence of 100 nM insulin, lysed, and subjected either to immunoblot analysis with antibodies to HA (A) or to immunoprecipitation with antibodies to FLAG (B and C). The immunoprecipitates were assayed for Akt kinase activity, and radioactivity incorporated into histone 2B was visualized (B) or quantitated (C) with an image analyzer. Data in panel C are means ± standard errors from three experiments.

precipitations with antibodies to HA, the amounts of Akt protein remaining in the supernatant were similar in infected and noninfected cells (Fig. 5A), indicating that Akt-AA was removed by this procedure. The insulin-induced activation of endogenous Akt was found to be inhibited by AxCAAkt-AA in an MOI-dependent manner, with 95% inhibition apparent at an MOI of 20 (Fig. 5B). In contrast, insulin-induced activation of MAP kinase was not affected by the expression of Akt-AA (Fig. 5C). Furthermore, infection of the cells with a control virus containing the *lacZ* gene (AxCALacZ) at an MOI of 20 had no effect on insulin-stimulated Akt activity (data not shown), suggesting that the inhibition of insulin-induced Akt activation by AxCAAkt-AA was not due to a nonspecific effect of viral infection.

We confirmed the dominant negative effect of Akt-AA with 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were infected with AxCAAkt-AA or AxCAΔp85 and incubated in the absence or presence of insulin. The activity of endogenous Akt was then assayed after immunodepletion of Akt-AA. As with CHO cells, three sequential immunoprecipitations with antibodies to HA removed Akt-AA (Fig. 6B). Infection of the adipocytes with AxCAΔp85 inhibited insulin-induced activation of endogenous Akt in an MOI-dependent manner, with ~65% inhibition apparent at an MOI of 30 (Fig. 6A). Infection of the cells with AxCAAkt-AA also inhibited insulin-induced activation of endogenous Akt, with ~80% inhibition apparent at an MOI of 200 (Fig. 6C). DNA laddering was not evident with 3T3-L1 adipocytes infected with AxCAAkt-AA at an MOI of 200 (data not shown). Infection of 3T3-L1 adipocytes with AxCAAkt-AA did not affect either the abundance of GLUT4 protein or morphological characteristics of the adipocytes (data not shown), suggesting that Akt-AA did not cause a change in phenotype of the adipocytes during the time course

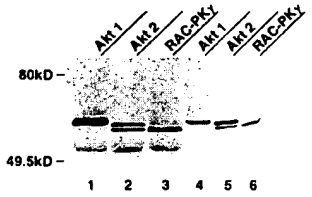

FIG. 4. Polyclonal antibodies to Akt recognize all three known isoforms of Akt. COS-7 cells cultured in 6-cm-diameter plates were transiently transfected with 3 μg of plasmid-encoded FLAG-tagged Akt1, Akt2, or RAC-PKγ. Lysates prepared from COS-7 cells expressing each isoform of Akt were subjected to immunoprecipitation with polyclonal antibodies to Akt. The immunoprecipitates (lanes 1 to 3) or the total cell lysates (lanes 4 to 6) were subjected to immunoblot analysis with antibodies to FLAG.

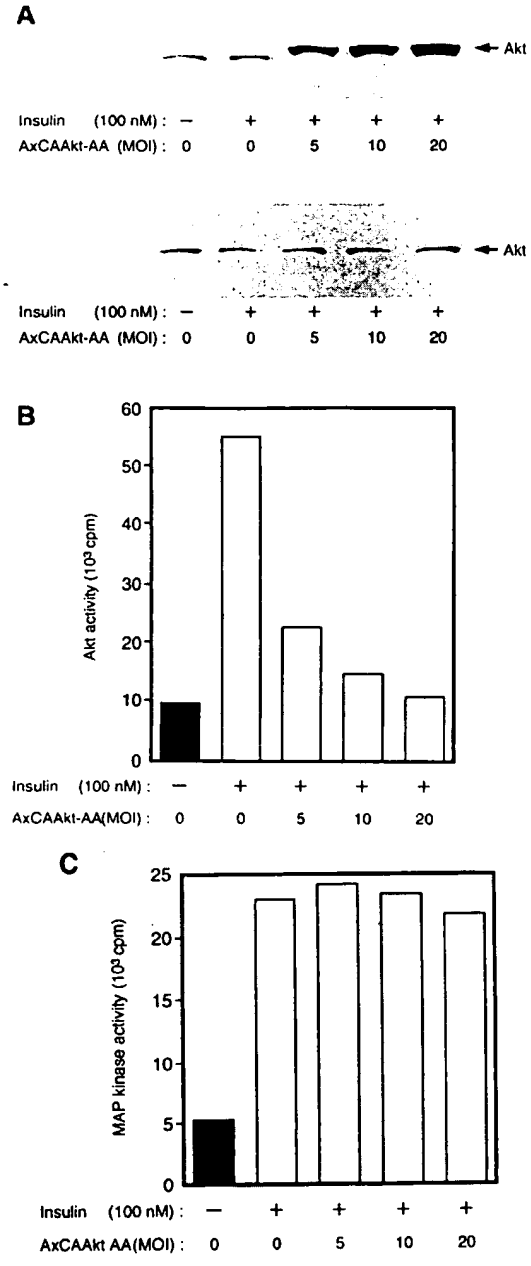

FIG. 5. Effects of Akt-AA on insulin-induced activation of endogenous Akt and MAP kinase in CHO cells. CHO cells cultured in 6-cm-diameter plates were infected with AxCAAkt-AA at the indicated MOI (PFU/cell), incubated for 10 min in the absence or presence of 100 nM insulin, and lysed. (A) Cell lysates were subjected either to immunoblot analysis with polyclonal antibodies to Akt (top) or to three sequential rounds of immunoprecipitation with antibodies to HA in order to deplete Akt-AA, after which the final supernatant was subjected to immunoblot analysis with polyclonal antibodies to Akt (bottom). (B and C) The Akt-AA-depleted final supernatant from panel A was subjected to immunoprecipitation with polyclonal antibodies to Akt (B) or with antibodies to MAP kinase (C), and the precipitates were assayed for Akt kinase or MAP kinase activity, respectively. Data are representative of three independent experiments, with bars representing means of duplicate determinations.

of the experiments. Thus, Akt-AA exerts a dominant negative effect in both CHO cells and 3T3-L1 adipocytes.

Effects of Akt-AA on insulin-stimulated protein synthesis in CHO cells and 3T3-L1 adipocytes. We next investigated the effect of Akt-AA on insulin-stimulated bulk protein synthesis, which has previously been shown to be regulated by PI 3-kinase (10, 34). Insulin stimulated an ~1.8-fold increase in protein synthesis in CHO cells within 1 h (Fig. 7A). Cells that had been exposed to wortmannin before treatment with insulin showed a level of protein synthesis that was less than the basal value. Infection of cells with AxCAAkt-AA inhibited insulin-stimulated protein synthesis in an MOI-dependent manner, without affecting the basal level; insulin-stimulated protein synthesis was completely abolished at an MOI of 20 PFU/cell.

Because wortmannin inhibits insulin-stimulated amino acid transport (54), we examined the effect of Akt-AA on this action of insulin. Insulin induced an ~1.2-fold increase in amino acid transport in CHO cells (Table 1), and treatment of cells with wortmannin before incubation with insulin reduced the extent of amino acid transport to below the basal value. Infection of the cells with AxCAAkt-AA at an MOI of 20 PFU/cell had no effect on insulin-stimulated amino acid transport, indicating that the inhibition of insulin-stimulated protein synthesis by Akt-AA was not attributable to an effect on amino acid transport.

In 3T3-L1 adipocytes, insulin induced an ~1.5-fold increase in protein synthesis within 1 h (Fig. 7B). Pretreatment of the cells with wortmannin abolished insulin stimulation of protein synthesis in these cells. As with CHO cells, infection of 3T3-L1 adipocytes with AxCAAkt-AA resulted in an MOI-dependent inhibition of insulin-stimulated protein synthesis. When CHO cells or 3T3-L1 adipocytes were infected with a control virus containing the *lacZ* gene at an MOI of 20 or 100, respectively, insulin-stimulated protein synthesis in these cells was not affected (data not shown). Furthermore, infection of AxCA ΔSOS at an MOI sufficient for almost complete inhibition of insulin-induced activation of MAP kinase activity in the adipocytes had no effect on insulin-induced protein synthesis (data not shown).

Effects of Akt-AA on insulin-stimulated p70 S6 kinase activity. We next investigated the effects of Akt-AA on insulin-induced activation of p70 S6 kinase, the activity of which has previously been shown to be increased as a result of overexpression of a membrane-targeted mutant Akt or a Gag-Akt fusion protein (4, 26). Insulin induced an approximately sixfold increase in p70 S6 kinase activity in CHO cells (Fig. 8A). Infection of the cells with AxCAAkt-AA inhibited insulin-stimulated activation of p70 S6 kinase in an MOI-dependent manner, with ~75% inhibition apparent at an MOI of 20 PFU/cell.

We also examined the effect of Akt-AA on insulin-stimulated p70 S6 kinase activity in 3T3-L1 adipocytes. Infection of the adipocytes with AxCAAkt-AA at an MOI of 200 PFU/cell, a dose that inhibited insulin-induced activation of endogenous Akt by ~80% (Fig. 6C), reduced the p70 S6 kinase activity promoted by 100 nM insulin by ~30% (Fig. 8B). More than 70% inhibition was apparent in the cells stimulated with 1 nM insulin, and ~40% inhibition was observed in those stimulated with 10 nM insulin (Fig. 8C).

Effects of Akt-AA on insulin-stimulated glucose uptake and GLUT4 translocation. Finally, we examined the effects of Akt-AA on insulin-stimulated glucose uptake. Insulin induced an approximately twofold increase in glucose uptake in CHO cells, an effect that was inhibited by infection of the cells with AxCAΔp85 (Fig. 9A), consistent with our previous data (16). However, infection of CHO cells with AxCAAkt-AA had no

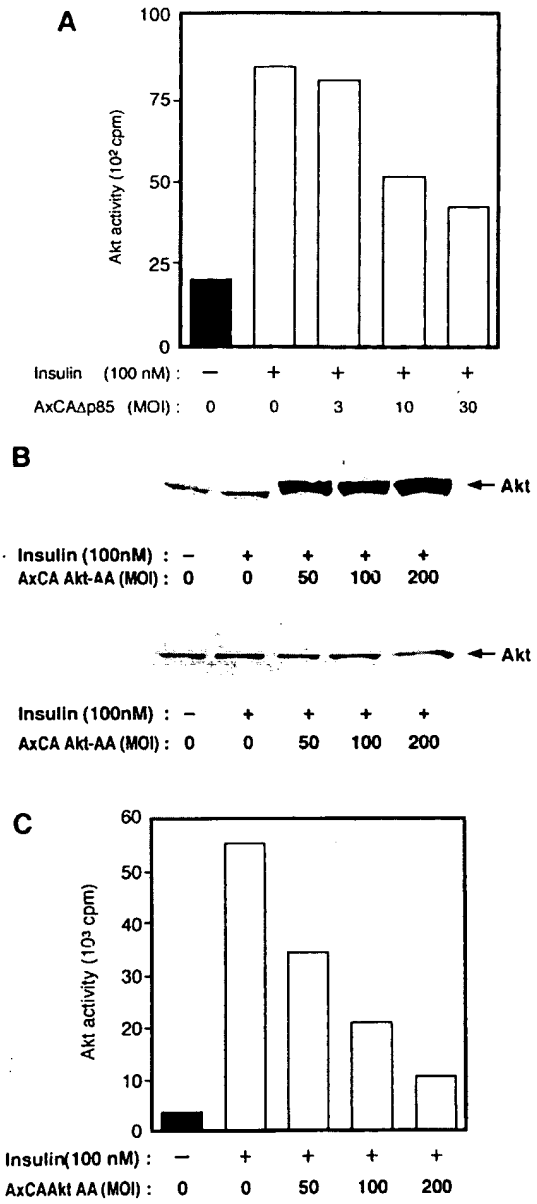

FIG. 6. Effects of Δp85 (A) and Akt-AA (B and C) on insulin-induced activation of endogenous Akt in 3T3-L1 adipocytes. 3T3-L1 adipocytes were infected with AxCAΔp85 (A) or AxCAAkt-AA (B and C) at the indicated MOI (PFU/cell), incubated for 10 min in the absence or presence of 100 nM insulin, and lysed. Cell lysates infected with AxCAAkt-AA were subjected either to immunoblot analysis with polyclonal antibodies to Akt (B, top) or to three sequential rounds of immunoprecipitation with antibodies to HA in order to deplete Akt-AA, after which the final supernatant was subjected to immunoblot analysis with polyclonal antibodies to Akt (B, bottom). Cell lysates infected with AxCAΔp85 or the Akt-AA-depleted final supernatant from panel B were subjected to immunoprecipitation with polyclonal antibodies to Akt; then these immunoprecipitates were assayed for Akt kinase activity. Data are representative of three independent experiments, with bars representing means of duplicate determinations.

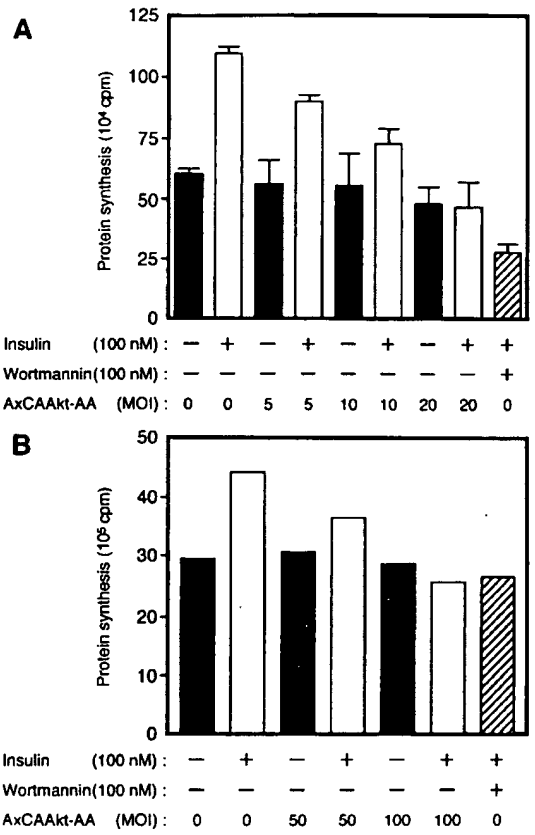

FIG. 7. Effects of Akt-AA on insulin-stimulated protein synthesis in CHO cells (A) and 3T3-L1 adipocytes (B). CHO cells or 3T3-L1 adipocytes were infected with AxCAAkt-AA at the indicated MOI (PFU/cell). The cells were incubated in the absence or presence of 100 nM wortmannin for 30 min, after which insulin-stimulated protein synthesis was assayed as described in Materials and Methods. Data are means ± standard errors from three experiments (A) or means of two experiments (B).

effect on insulin-stimulated glucose uptake (Fig. 9B), even at a virus concentration (MOI of 20 PFU/cell) that inhibited insulin-induced activation of endogenous Akt by ~95% (Fig. 5B). Wortmannin abolished insulin stimulation of glucose uptake in cells that had been infected with AxCAAkt-AA (data not shown).

Constitutively active Akt mutant proteins have been shown to stimulate glucose uptake and translocation of GLUT4 in adipocytes (27, 53). We therefore examined the effects of Akt-AA on glucose uptake and GLUT4 translocation in 3T3-L1 adipocytes. Insulin induced an approximately eightfold

TABLE 1. Effect of Akt-AA on insulin-stimulated amino acid uptake in CHO cells[a]

Condition	MeAIB uptake (cpm) at AxCAAkt-AA MOI of:	
	0	20
Basal	1,154 ± 109	1,120 ± 60
Insulin (100 nM)	1,387 ± 20	1,401 ± 41
Wortmannin (100 nM) + insulin (100 nM)	867 ± 19	

[a] CHO cells cultured in six-well plates were infected or not with AxCAAkt-AA at an MOI of 20 PFU/cell and incubated in the absence or presence of 100 nM wortmannin for 20 min. Insulin-stimulated MeAIB transport into the cells was then assayed as described in Materials and Methods. Data are means ± standard errors from three experiments.

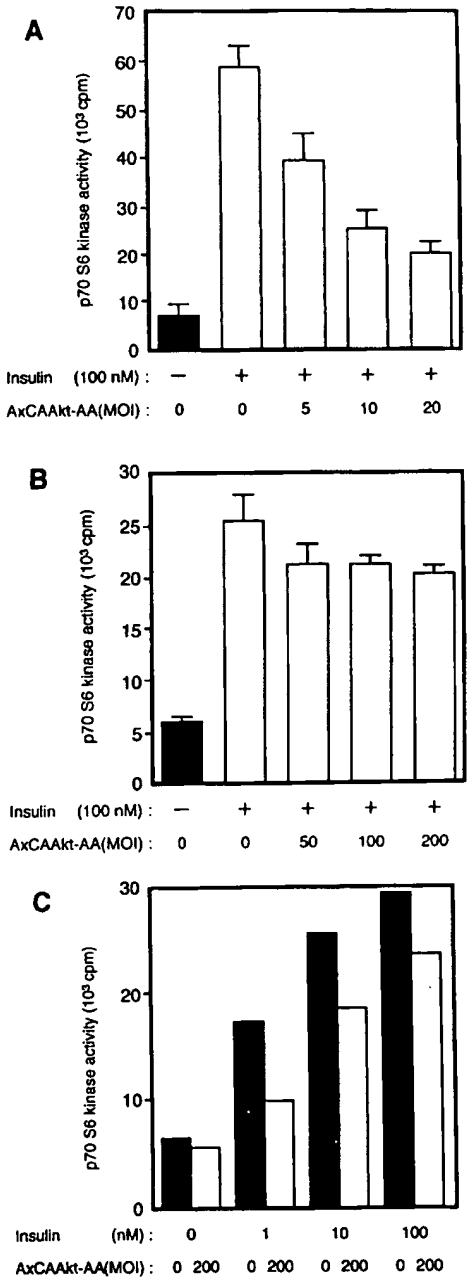

FIG. 8. Effects of Akt-AA on insulin-induced activation of p70 S6 kinase in CHO cells (A) and 3T3-L1 adipocytes (B and C). Cells were infected with AxCAAkt-AA at the indicated MOI (PFU/cell), incubated in the absence or presence of the indicated concentrations of insulin for 10 min, lysed, and subjected to immunoprecipitation with antibodies to p70 S6 kinase. The resulting immunoprecipitates were assayed for p70 S6 kinase activity as described in Materials and Methods. Data are means ± standard errors from three experiments (A and B) or means of two experiments (C).

increase in glucose uptake in these cells. Consistent with our previous results (48), AxCAΔp85 inhibited insulin-stimulated glucose uptake in an MOI-dependent manner (Fig. 9C). In contrast, AxCAAkt-AA had no effect on glucose uptake (Fig. 9D) even at an MOI of 200. Furthermore, a dose-response curve of insulin-stimulated glucose uptake in the cells infected with AxCAAkt-AA at an MOI of 200 was similar to that of noninfected cells (Fig. 9E). We examined the effect of Akt-AA on insulin-induced translocation of GLUT4 by the plasma membrane lawn assay. Whereas plasma membrane lawns prepared from quiescent adipocytes showed little GLUT4 immunoreactivity, insulin induced a marked increase in the amount of the glucose transporter in the plasma membrane (Fig. 10A and B). Infection of the cells with AxCAΔp85 inhibited the insulin-induced increase in GLUT4 immunoreactivity in the membrane (Fig. 10C), whereas AxCAAkt-AA had no effect on this action of insulin (Fig. 10D).

DISCUSSION

We have investigated the roles of Akt in cells by specifically inhibiting the activity of the endogenous enzyme. Such inhibition was achieved by expression of a mutant Akt (Akt-AA) in which the sites of ligand-induced phosphorylation are mutated to alanine and that acts in a dominant negative manner. Akt-AA was introduced into both CHO cells and 3T3-L1 adipocytes with the use of an adenovirus expression system, which has previously been used to express a variety of genes with high efficiency (36). The maximal abundance of Akt protein in CHO cells or 3T3-L1 adipocytes infected with AxCAAkt-AA was 30 to 50 times that of endogenous Akt.

We tested several mutants of Akt for dominant negative effects, including a protein in which Lys^{179} in the kinase domain was replaced by asparate (Akt-K179D). Although Akt-K179D did not exhibit kinase activity, it was less effective than Akt-AA in inhibiting the activity of endogenous Akt when overexpressed by the adenovirus system. Activity-deficient mutants of protein kinases in which phosphorylation sites targeted by extracellular stimuli have been replaced by neutral amino acids have previously been shown to act in a dominant negative manner. For example, mutants of MAP kinase in which either Thr^{192} or Tyr^{194} is replaced by alanine (40) act in a dominant negative manner. Furthermore, a MEK protein in which extracellular stimulus-dependent phosphorylation sites were changed to alanine also behaved as a dominant negative mutant (8). Such a mutant MEK showed increased binding to Raf, a kinase upstream of MEK, compared with wild-type MEK (60). These data, together with our observation that Akt-K179D is phosphorylated in vivo in response to insulin (unpublished data) and by a putative upstream kinase in vitro (51), suggest that Akt-AA may interact with its upstream kinase with higher affinity than does either wild-type Akt or Akt-K179D and that this higher-affinity interaction underlies its dominant negative effects.

Overexpression of a membrane-targeted mutant Akt or a Gag-Akt fusion protein, the kinase activity of both of which is greater than that of Akt-WT, was previously shown to increase glucose uptake or translocation of GLUT4 in quiescent adipocytes (27, 53). However, we have now shown that inhibition of endogenous Akt activity by Akt-AA had no effect on glucose transport. The simplest explanation for these observations would be that Akt is not necessary for insulin-stimulated glucose uptake, although activated Akt is sufficient to increase glucose uptake under certain conditions. A constitutively active mutant of Ras also increases glucose uptake and translocation of GLUT4 (32, 45), whereas Ras activation and its downstream signaling are not required for insulin stimulation of glucose uptake (12, 17, 45, 48).

It is possible that the inhibition of endogenous Akt activity by overexpression of Akt-AA is not sufficient to affect insulin-stimulated glucose uptake. However, the same extent of inhi-

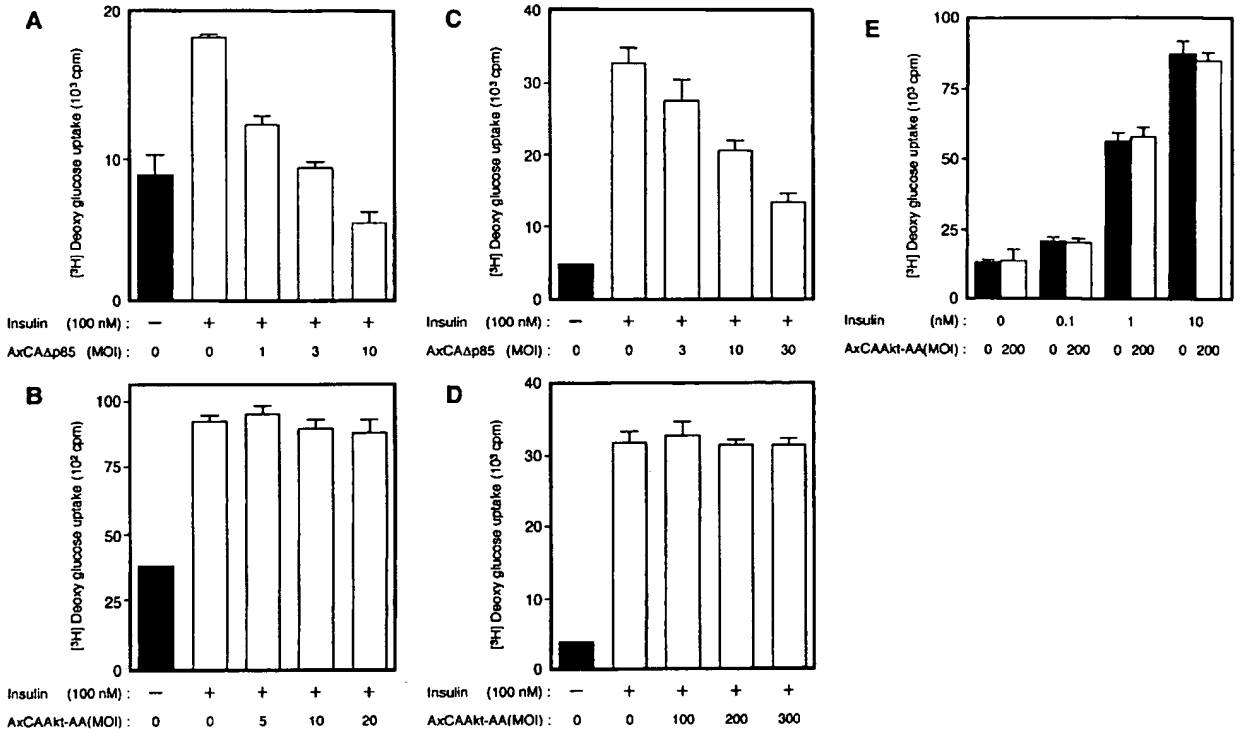

FIG. 9. Effects of Δp85 or Akt-AA on insulin-stimulated glucose uptake in CHO cells (A and B) and 3T3-L1 adipocytes (C to E). Cells were infected with AxCAΔp85 (A and C) or AxCAAkt-AA (B, D, and E) at the indicated MOI (PFU/cell), and insulin-stimulated glucose uptake was assayed as described in Materials and Methods. Data are means ± standard errors from three experiments.

bition of insulin-stimulated Akt activity achieved by a dominant negative mutant of PI 3-kinase (Δp85) was sufficient to inhibit insulin-induced glucose transport. The polyclonal antibodies used to examine endogenous Akt activity are capable of precipitating all three known isoforms of Akt (Akt1 [RAC-PKα], Akt2 [RAC-PKβ], and RAC-PKγ). Thus, our data indicate that the activities of all three Akt isoforms are inhibited by Akt-AA. However, we cannot exclude the possibility that an

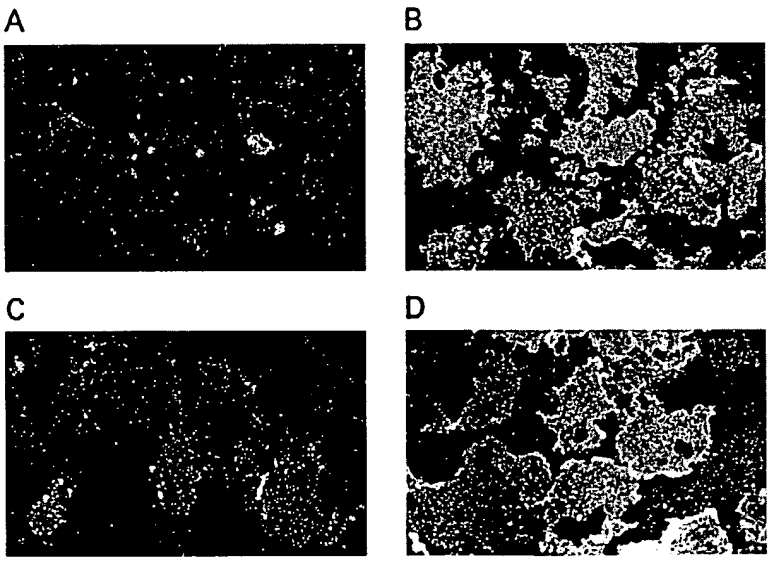

FIG. 10. Effects of Δp85 and Akt-AA on insulin-induced translocation of GLUT4 to the plasma membrane of 3T3-L1 adipocytes. Cells were not infected (A and B) or infected with AxCAΔp85 (C) or AxCAAkt-AA (D) at an MOI of 30 or 200 PFU/cell, respectively. The cells were incubated in the absence (A) or presence (B to D) of 100 nM insulin for 10 min, after which plasma membrane fragments were prepared and subjected to immunofluorescence microscopy with antibodies to GLUT4 and tetramethyl rhodamine isothiocyanate-labeled secondary antibodies. Data are representative of three independent experiments. Magnification, ×400.

unidentified isoform of Akt that is resistant to Akt-AA is present in the cells studied and responsible for insulin-stimulated glucose uptake.

We have shown that Akt-AA inhibited insulin-stimulated p70 S6 kinase activity, suggesting that Akt is required for activation of p70 S6 kinase. It is not clear why insulin-induced activation of p70 S6 kinase in CHO cells was inhibited by only ~75% whereas insulin activation of endogenous Akt was almost completely abolished in these cells. However, previous evidence has suggested that growth factor stimulation of p70 S6 kinase is mediated by redundant signaling pathways (43, 58). Thus, interruption of only one pathway (the Akt pathway) may be insufficient for complete inhibition of p70 S6 kinase activation. The relatively small inhibitory effect of Akt-AA on insulin activation of p70 S6 kinase in 3T3-L1 adipocytes may reflect a minor contribution of Akt to this action of insulin in these cells.

Wortmannin inhibits insulin-stimulated bulk protein synthesis (10, 34). Furthermore, we have recently shown that a dominant negative mutant of PI 3-kinase inhibited insulin-stimulated protein synthesis in CHO cells (52a). These data suggest that PI 3-kinase is important for insulin-stimulated bulk protein synthesis. However, it has not been known at which of the multiple steps of protein synthesis (21) PI 3-kinase exerts its effect. Tsakiridis et al. (54) showed that wortmannin inhibits insulin-stimulated amino acid transport in L6 myoblast cells. We have now shown that Akt-AA inhibited protein synthesis but not amino acid transport in CHO cells. These data demonstrate that the Akt pathway affects protein synthesis at a step distinct from amino acid transport. However, we cannot exclude the possibility that upstream kinases of Akt have physiological substrates other than Akt and that the dominant negative effects of Akt-AA are, at least in part, due to the inhibition of phosphorylation of these substrates but not of Akt.

p70 S6 kinase is thought to contributes to the regulation of protein synthesis by phosphorylating ribosomal protein S6 (43). Phosphorylation of S6 in intact cells correlates with increased protein synthesis (21). However, the inhibitory effect of Akt-AA on bulk protein synthesis is probably not due to the inhibition of p70 S6 kinase because rapamycin, which prevents insulin activation of p70 S6 kinase (41, 43), has only a small effect on insulin-stimulated protein synthesis (10, 34). We recently showed that insulin-induced activation of guanine nucleotide exchange activity toward translation initiation factor eIF-2 in total lysates of CHO-IR cells was completely inhibited by overexpression of a dominant negative PI 3-kinase (57). The eIF-2B factor is thought to mediate this guanine nucleotide exchange activity and subsequently to regulate recruitment of the initiator Met-tRNA to the 40S ribosomal subunit (46), an essential step for initiation of translation. Glycogen synthase kinase-3β (GSK-3β), a putative downstream effector of Akt (9), has been suggested to participate in the regulation of eIF-2B (56). Although the effects of Akt-AA on regulation of GSK-3β and eIF-2B remain to be elucidated, Akt-AA may affect bulk protein synthesis by inhibiting the GSK-3β–eIF-2B pathway.

In summary, we have identified a dominant negative mutant of Akt and, with the use of an adenovirus encoding this protein, shown that Akt mediates some, but not all, signaling pathways downstream of PI 3-kinase. Because PI 3-kinase contributes not only to the metabolic actions of insulin but to a variety of biological effects, it will be important to determine which other signaling pathways downstream of PI 3-kinase are mediated through Akt. An atypical PKC isozyme, PKCζ, is a putative downstream effector of PI 3-kinase (38) and has recently been shown to be required for insulin-stimulated bulk protein synthesis (35). It is not clear which steps of protein synthesis are regulated by PKCζ, and so it remains to be determined whether Akt and PKCζ participate in the same signaling pathway or whether each controls protein synthesis by regulating different steps.

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Construction and Characterization of a Conditionally Active Version of the Serine/Threonine Kinase Akt*

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Akt is a serine/threonine kinase that requires a functional phosphatidylinositol 3-kinase to be stimulated by insulin and other growth factors. When directed to membranes by the addition of a *src* myristoylation sequence, Akt becomes constitutively active. In the present study, a conditionally active version of Akt was constructed by fusing the Akt containing the myristoylation sequence to the hormone binding domain of a mutant murine estrogen receptor that selectively binds 4-hydroxytamoxifen. The chimeric protein was expressed in NIH3T3 cells and was shown to be stimulated by hormone treatment 17-fold after only a 20-min treatment. This hormone treatment also stimulated an approximate 3-fold increase in the phosphorylation of the chimeric protein and a shift in its migration on SDS gels. Activation of this conditionally active Akt resulted in the rapid stimulation of the 70-kDa S6 kinase. This conditionally active Akt was also found to rapidly stimulate in these cells the phosphorylation of properties of PHAS-I, a key protein in the regulation of protein synthesis. The conditionally active Akt, when expressed in 3T3-L1 adipocytes, was also stimulated, although its rate and extent of activation was less then in the NIH3T3 cells. Its stimulation was shown to be capable of inducing glucose uptake into adipocytes by stimulating translocation of the insulin-responsive glucose transporter GLUT4 to the plasma membrane.

Akt is a serine/threonine kinase that contains a pleckstrin homology (PH)[1] domain at its amino terminus (1, 2). The PH domain is a protein module found in many signal transduction proteins that can mediate either protein-lipid or protein-protein interactions (1, 2). The kinase activity of Akt is stimulated by a number of different growth factors, including insulin and platelet-derived growth factor (3–5). Several studies have shown that Akt stimulation requires prior activation of phosphatidylinositol 3-kinase (PI 3-kinase) (3–5), possibly due to a direct interaction of the Akt PH domain with these lipids products (6) or, alternatively, due to the phosphorylation of Akt by a distinct Ser/Thr kinase, which is activated by PI 3-phosphates (7).

Further interest in Akt has been stimulated by the finding that this enzyme can induce a variety of biological responses. In particular, Akt has been proposed to positively regulate the 70-kDa S6 kinase (4) and negatively regulate the GSK-3 kinase (8). Moreover, Akt has been shown to be capable of stimulating the differentiation of 3T3-L1 cells into adipocytes (9, 10) and to inhibit apoptosis of neuronal cells, as well as fibroblasts (11, 12). Finally, Akt has been shown to stimulate lipogenesis (9) and to induce glucose uptake into adipocytes by stimulating GLUT4 translocation to the plasma membrane (9, 13).

To determine whether a particular biological response can be mediated via Akt, the above studies made use of a constitutively active form of Akt in which the enzyme is targeted to membranes via the addition of either the src myristoylation signal or a myristoylated gag sequence (4, 8–13). Such studies have the problem that the Akt kinase activity is unregulated, and thus the kinase is active as soon as it is expressed in cells. In contrast, the responses one is attempting to mimic, such as stimulation of glucose uptake, are stimulated by insulin within minutes. In addition, such studies require one to utilize different populations of cells to compare a particular response; for example, cells expressing the constitutively active Akt must be compared with control cells.

In this report, we describe and characterize a conditionally active form of the Akt molecule. This conditionally active form of Akt was created by fusing the hormone binding domain (HBD) of a mutant murine estrogen receptor (14) to a variant of Akt that lacks its PH domain but contains a *src* myristoylation signal at its amino terminus (myrAkt Δ4–129) (15). The Akt construct without the HBD has unregulated constitutive kinase activity (15). This mutant HBD of the estrogen receptor, which has also been used to make a conditionally active myc (14), does not bind 17β-estradiol but is responsive to 4-hydroxytamoxifen (14). In the present work, we show that the kinase activity of the Akt-estrogen receptor fusion protein (myrAkt Δ4–129-ER) is dependent on 4-hydroxytamoxifen (HT). This conditionally active form of Akt was used to demonstrate that acute activation of Akt was sufficient to stimulate the phosphorylation of PHAS-I (phosphorylated heat- and acid-stable protein) and to induce its dissociation from eIF4E, a key step in the regulation of protein synthesis.

EXPERIMENTAL PROCEDURES

Constructs—To make an estradiol-dependent human Akt protein kinase, the hormone binding domain of a mutant murine estrogen

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[1] The abbreviations used are: PH, pleckstrin homology; PI, phosphatidylinositol; HT, hydroxytamoxifen; ER, estrogen receptor; HBD, hormone binding domain; HA, hemagglutinin.

receptor (ER) (a gift from Dr. Martin McMahon) that no longer binds 17β-estradiol but is activated by the synthetic steroid 4-hydroxytamoxifen (14) was first subcloned into the pWZLneo retroviral vector (16), which carries a neomycin resistance gene, using the *Eco*RI and *Sal*I restriction sites at the 5′- and 3′-ends, respectively. In addition, the polymerase chain reaction was used to modify myrAkt Δ4–129 and A2myrAkt Δ4–129 (15) by replacing the stop codon after the hemagglutinin (HA) epitope tag with an in-frame *Eco*RI site. The fragment obtained by the polymerase chain reaction was confirmed by sequencing. The Akt constructs were then fused to the estrogen receptor in pWZLneo using the 5′ *Bam*HI and 3′ *Eco*RI restriction sites, resulting in the formation of myrAkt Δ4–129-ER and A2myrAkt Δ4–129-ER.

A p70 S6 kinase clone (a gift from Dr. Gerry Crabtree) was modified to encode a myc epitope-tag at its 5′ end and subcloned into the pWZLneo retroviral vector using standard molecular biological techniques.

Retroviral Infection—NIH3T3 fibroblasts and 3T3-L1 preadipocytes were infected with either myrAkt Δ4–129-ER or A2myrAkt Δ4–129-ER as described previously (15).

NIH3T3 cells infected with either myrAkt Δ4–129-ER or A2myrAkt Δ4–129-ER were then infected with the pWZLneo retroviral vector expressing the myc epitope-tagged p70 S6 kinase construct. The cells were infected using the same protocol noted above, except that the cells were not subjected to additional drug selection.

Akt, p70 S6, PI 3-Kinase Assays, PHAS-I Shift, and eIF4E Dissociation—The Akt immunoprecipitations and immunoblotting were performed as described previously (9), except that a single 100-mm plate was used for each treatment, and each plate was lysed in 400 μl of lysis buffer. In most experiments, a peptide (sequence GRPRTSSFAEG) assay was utilized to measure Akt kinase activity as described (8), except that a 40% SDS-polyacryamide gel was utilized to separate the incorporated label from free ATP. This assay was linear for up to 90 min, and all the values determined were within the linear range for Akt levels. To detect phosphorylation of Ser^{473}, immunoblotting was performed with the phospho-specific Akt antibody from New England BioLabs.

The expressed p70 S6 kinase was immunoprecipitated using a monoclonal antibody directed against the myc epitope tag (Babco) that was preadsorbed to protein G-Sepharose. The nonspecific background was measured by incubating lysates with normal mouse immunoglobulin that was also preadsorbed to protein G-Sepharose. The kinase activity was measured as described previously (15). 3× Laemmli sample buffer (45 μl) was also added to the remaining beads. The bound protein was eluted by incubating for 4 min at 100 °C, and these samples were electrophoresed on 10% SDS-polyacrylamide gels. The gels were transferred and immunoblotted using the anti-myc antibody to detect p70 S6 kinase.

The PI 3-kinase assay using pure phosphatidylinositol (Sigma) as a substrate was performed as described previously (17), except that cells were serum-starved for 16 h before being treated and lysed. The PHAS-I shift and eIF4E dissociation were measured by PHAS-I immunoblotting either whole cell extracts and m^7GTP-Sepharose bound material, respectively, as described previously (18).

3T3-L1 Preadipocytes and Adipocytes—3T3-L1 preadipocytes infected with either myrAkt Δ4–129-ER or A2myrAkt Δ4–129-ER were cultured, selected, and differentiated as described previously (9). These cell lines were used to measure glucose uptake and to isolate crude membrane fractions for detection of GLUT1 and GLUT4 expression as described previously (9). Translocation of GLUT4 to the plasma membrane was performed and quantitated as described previously (19, 20).

In Vivo Labeling of myrAkt Δ4–129-ER—Medium was replaced with Krebs-Ringer bicarbonate buffer containing 10 mM glucose and [^{32}P]orthophosphate (500 μCi/plate). After 3 h at 37C/5%CO_2, NIH3T3 cells expressing myrAkt Δ4–129-ER were treated without or with 1 μM HT for additional 40 min, placed on ice, washed with ice-cold HBS, and lysed with 500 μl of lysis buffer (50 mM Hepes, pH 7.4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 300 nM okadaic acid, 1 mM NaF, 10 mM β-glycerol phosphate, 10 μg/ml aprotinin). Lysates were immunoprecipitated with monoclonal anti-HA (12CA5) bound to Protein-A agarose beads. Beads were washed, and bound proteins were eluted and analyzed by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. To confirm which band was Akt, the membrane was immunoblotted with anti-HA antibodies. The autoradiographs were scanned, imported into Adobe Photoshop, and quantitated after subtracting a background region of the gel.

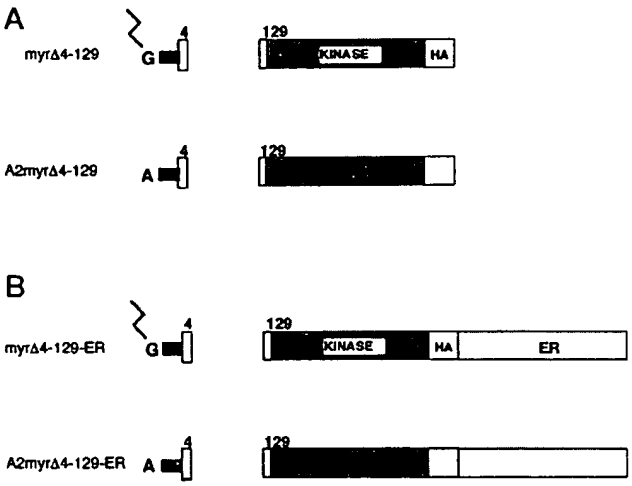

FIG. 1. **Schematic representation of the Akt constructs described in this study.** *A*, unregulated Akt alleles: Akt constructs described previously (9) that bear a myristoylation signal sequence (myr) at the amino terminus lack the amino-terminal pleckstrin homology domain (Δ4–129) and possess a carboxyl-terminal HA epitope tag. The kinase activity of myrAkt Δ4–129 is constitutive and unregulated. *B*, conditional Akt alleles: conditionally active Akt alleles that are fused at their carboxyl terminus to the hormone binding domain of an ER. The conditional Akt alleles were generated as described under "Experimental Procedures."

RESULTS

Production of a Conditionally Active Akt Kinase—Several strategies exist to make a conditionally active Akt kinase, the activity of which can be regulated. Tetracycline-regulatable systems have been described in which tetracycline can be added to suppress or induce expression of a given construct (21). In addition, one could use drug-induced dimerization systems exploiting synthetic bivalent membrane permeable compounds that bind to drug binding domains fused to intracellular signaling molecules (22). Finally, conditionally active forms of the transcription factor Myc and the protein kinases Raf1 and Abl have been made by fusing them with the HBD of steroid receptors, particularly the estrogen receptor (23–25). Although we were unable to create a conditionally active form of Akt using the tetracycline-regulatable or drug-induced dimerization systems (data not shown), we were able to generate such an enzyme by constructing a HBD fusion protein.

We have previously described a constitutively active form of Akt (myrAkt Δ4–129) created by attaching the *src* myristoylation signal to the amino terminus of a variant of Akt that lacked its PH domain and carried a HA epitope tag at its carboxyl terminus (15). We also described a control construct (A2myrAkt Δ4–129) that was not constitutively active in which the myristoylated glycine at the second amino acid position was converted to alanine to eliminate the membrane-targeting function of the *src* myristoylation signal sequence (15). To generate a conditionally active form of Akt, the myristoylated Akt construct and the mutant control lacking the myristoylated glycine were fused in-frame to the HBD of a mutant form of the murine estrogen receptor (myrAkt Δ4–129-ER and A2myrAkt Δ4–129-ER) (Fig. 1) that no longer binds 17β-estradiol but is still responsive to the synthetic steroid HT (26). These fusion proteins were subcloned into a retroviral vector that carried a neomycin resistance gene (16).

NIH3T3 cells were infected with retroviruses encoding the different forms of Akt, drug-selected, serum-starved for 16 h, and then treated for increasing amounts of time with HT. The Akt fusion protein was immunoprecipitated from cell lysates using a monoclonal antibody directed against the HA tag, and

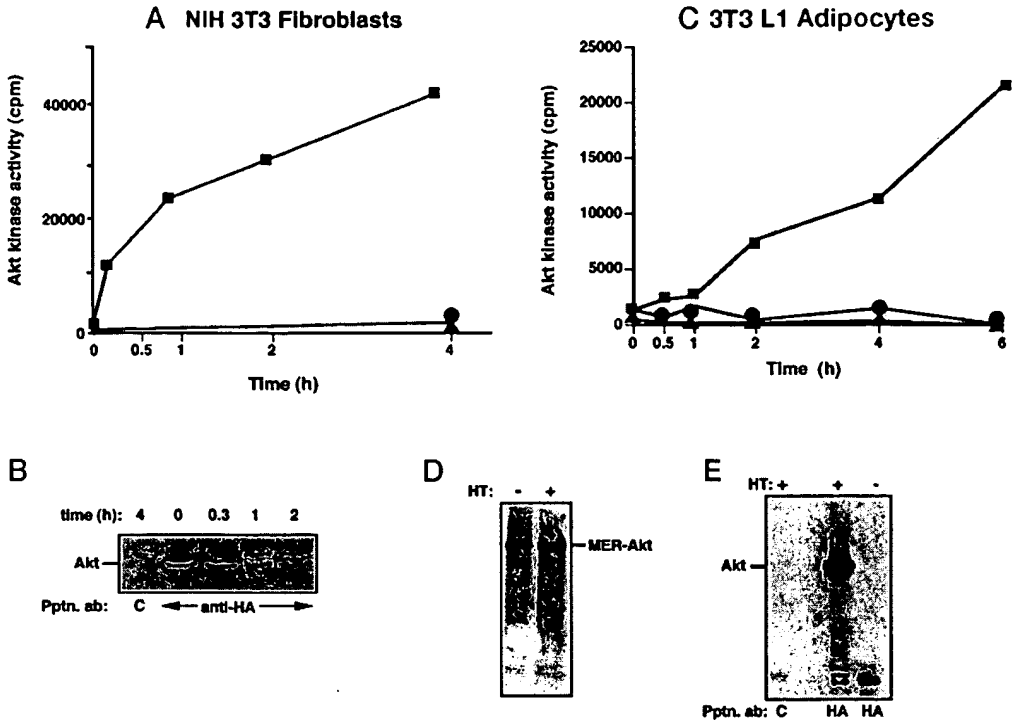

FIG. 2. **Activation of the conditionally active Akt.** Time course of activation in NIH3T3 cells (*A*) and 3T3-L1 adipocytes (*C*). NIH3T3 cells expressing either myrAkt Δ4–129-ER (■) or A2myrAkt Δ4–129-ER (●) were treated for the indicated amounts of time with 1 μM HT and lysed, the chimera was immunoprecipitated, and its enzymatic activity was measured *in vitro* using a GSK-3 peptide as substrate. A control of the myrAkt Δ4–129-ER treated with control vehicle (ethanol) is also shown (▲). *B*, HT-induced shift in Akt. The immunoprecipitated myrAkt Δ4–129-ER from NIH3T3 cells treated for the indicated periods of time with HT were separated on a 10% SDS-polyacrylamide gel, and the Akt protein was detected by Western blot using a polyclonal anti-HA antibody. A control immunoprecipitate using normal mouse Ig is also shown (*lane C*). *D*, HT-induced phosphorylation of myrAkt Δ4–129-ER. Metabolically labeled NIH3T3 cells expressing myrAkt Δ4–129-ER were treated or not treated with 1 μM HT for 40 min and lysed, and the lysates were immunoprecipitated with the anti-HA antibodies. *E*, HT-induced phosphorylation of Ser^{473} in myrAkt Δ4–129-ER. NIH3T3 cells expressing myrAkt Δ4–129-ER were treated or not treated with 1 μM HT and lysed, and the lysates were immunoprecipitated with the anti-HA antibodies (*HA*). A control immunoprecipitate using normal mouse Ig is also shown (*lane C*). The precipitates were electrophoresed and blotted with a phospho-specific Akt antibody.

the kinase activity was measured *in vitro* using a synthetic peptide as substrate (8). Exposure of cells to HT for only 20 min caused the kinase activity of myrAkt Δ4–129-ER to increase 17-fold. The kinase activity continued to increase with longer exposures to HT to more than a 50-fold activation (Fig. 2*A*). In contrast, the kinase activity of myrAkt Δ4–129-ER treated with ethanol alone and the activity of A2myrAkt Δ4–129-ER treated with either ethanol or HT was barely elevated compared with control immunoprecipitates using normal mouse immunoglobulin.

The immunoprecipitated protein was also analyzed by immunoblotting using antibody directed against the HA epitope. Treatment of cells with HT did not change the expression level of myrAkt Δ4–129-ER. However, HT treatment was associated with the appearance of a slower migrating band (Fig. 2*B*), consistent with an increase in the phosphorylation state of the protein.

Studies on the Mechanism of Activation of the Conditionally Active Akt—As shown above, although the levels of the myrAkt Δ4–129-ER protein did not change after HT treatment of cells, the protein did shift to a higher molecular weight on SDS gels (Fig. 2*B*), consistent with an increase in phosphorylation. To directly test this, metabolically labeled cells expressing myrAkt Δ4–129-ER were treated for 40 min with either HT or ethanol and lysed, and the lysates were immunoprecipitated with anti-HA antibodies. An approximate 3-fold increase (range, 1.8–3.4; $n = 3$) in labeling of the myrAkt Δ4–129-ER was observed with HT treatment (Fig. 2*D*).

To determine whether the activating site Ser^{473} was phosphorylated, the immunoprecipitated myrAkt Δ4–129-ER was blotted with a phospho-specific antibody directed against this site. HT treatment greatly stimulated the ability of the phosphospecific antibody to recognize this chimera (Fig. 2*E*), demonstrating that HT treatment stimulates an increase in the phosphorylation of Ser^{473}.

To determine whether PI 3-kinase had any role in the activation of the conditionally active form of Akt, NIH3T3 cells that had been infected with myrAkt Δ4–129-ER were treated for 20 min with increasing concentrations of the PI 3-kinase inhibitor wortmannin (27). These cells were then treated for 10 min with HT to induce the Akt kinase activity. The basal PI 3-kinase activity was measured in anti-p85 antibody precipitates prepared from nonstimulated cells to verify the inhibitory effect of wortmannin. MyrAkt Δ4–129-ER was immunoprecipitated from cell lysates prepared from cells that had been treated with HT, and its kinase activity was measured *in vitro* to determine whether its activation was dependent on PI 3-kinase. At 1 nM wortmannin, a slight increase in Akt activity was observed, similar to a slight increase in Akt activity previously observed with 1 nM wortmannin after insulin treatment (5). However, at higher wortmannin concentrations, the activation of myrAkt Δ4–129-ER was inhibited, and this paralleled the inhibition of PI 3-kinase activity (Fig. 3). A structurally unrelated inhibitor of PI 3-kinase, LY294002 (28), was also found to block the activation of myrAkt Δ4–129-ER (data not shown).

The Conditionally Active Akt Rapidly Stimulates p70 S6 Kinase—Prior studies have shown that constitutively active forms of Akt stimulate the enzymatic activity of p70 S6 kinase

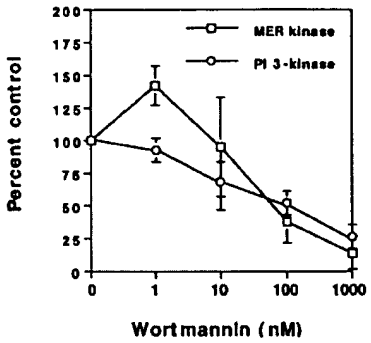

FIG. 3. **Activation of myrAkt Δ4–129-ER is inhibited by wortmannin.** To test the effect of wortmannin on myrAkt Δ4–129-ER activation, NIH3T3 cells expressing myrAkt Δ4–129-ER (*MER*) were treated with either control vehicle or increasing concentrations of wortmannin for 20 min, after which the cells were treated for 10 min with 1 μM HT. After the cells were lysed, the cell lysates were adsorbed with either monoclonal anti-HA antibody 12CA5 or normal mouse Ig and assayed for kinase activity using myelin basic protein as a substrate, as described under "Experimental Procedures." To measure PI 3-kinase activity, PI 3-kinase was immunoprecipitated from NIH3T3 cells infected with myrAkt Δ4–129-ER that had also been treated with either control vehicle or increasing concentrations of wortmannin for 20 min. PI 3-kinase was isolated using an antibody directed against the 85-kDa regulatory subunit, and its activity was measured as described under "Experimental Procedures." The values obtained were normalized to that obtained in the absence of wortmannin (100%) and are means ± S.E. (n = 3).

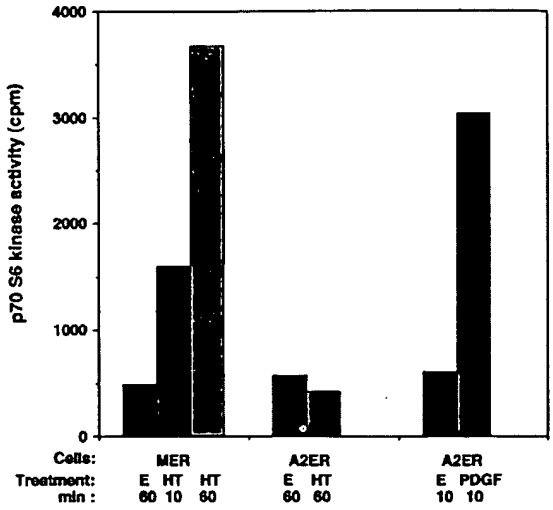

FIG. 4. **The effect of myrAkt Δ4–129-ER on p70 S6-kinase activity.** NIH3T3 cells expressing either myrAkt Δ4–129-ER (*MER*) or A2myrAkt Δ4–129-ER (*A2ER*) were infected with a retroviral vector encoding a myc-epitope-tagged p70 S6 kinase. After cells were serum-starved for 16 h, cells were treated for 10 or 60 min with either the control vehicle, ethanol (*E*), or 1 μM HT to induce Akt kinase activity. The p70 S6 kinase was immunoprecipitated using either an anti-myc monoclonal antibody or control normal mouse Ig. The p70 S6 kinase activity was measured *in vitro* using the 40 S ribosomal subunit as substrate, as described under "Experimental Procedures." A control with 2 nM platelet-derived growth factor stimulation was also included for the A2myrAkt Δ4–129-ER chimera.

(4, 15). In these reports, the constitutively active Akt was unregulated, so its kinase activity was chronically elevated, raising the possibility that p70 S6 kinase was stimulated not because of a phosphorylation cascade initiated by Akt but rather because of other cellular events arising secondary to long-term expression of a constitutively active kinase. The conditionally active Akt allowed us to resolve this question.

NIH3T3 cells already expressing either myrAkt Δ4–129-ER or A2myrAkt Δ4–129-ER were subsequently infected with a myc-epitope-tagged p70 S6 kinase that had also been subcloned into a retroviral vector. The cells were serum-starved for 16 h and then treated for 10 min with HT or with ethanol, the control vehicle. The p70 S6 kinase was immunoprecipitated using a monoclonal antibody directed against the myc epitope tag, and its kinase activity was measured *in vitro* using the 40S ribosomal subunit as a substrate. Induction of Akt kinase activity by treating cells that co-expressed myrAkt Δ4–129-ER with HT was associated with a 3- and 7-fold increase in p70 S6 kinase activity after a 10- and 60-min stimulation, respectively (Fig. 4). In contrast, treating cells that co-expressed A2myrAkt Δ4–129-ER with HT did not cause an increase in p70 S6 kinase activity compared with control-treated cells (Fig. 4). The stimulation of p70 S6 kinase after 60 min with HT in cells coexpressing myrAkt Δ4–129-ER chimera was comparable to the stimulation achieved using 2 nM platelet-derived growth factor (Fig. 4). The increased activity of p70 S6 kinase observed in the context of the induced myrAkt Δ4–129-ER was also associated with the appearance of hyperphosphorylated forms of p70 S6 kinase (data not shown), consistent with prior studies of this enzyme (29, 30).

The Conditionally Active Akt Stimulates Glucose Uptake and GLUT4 Translocation in 3T3-L1 Adipocytes—The unregulated constitutively active Akt has previously been shown to stimulate glucose uptake in 3T3-L1 adipocytes and induce the translocation of GLUT4 to the plasma membrane (9, 13). To test whether the conditionally active Akt could also stimulate this response, we infected 3T3-L1 preadipocytes with either myrAkt Δ4–129-ER or A2myrAkt Δ4–129-ER. The kinase activity of the Akt fusion proteins was measured in immunoprecipitates prepared from differentiated adipocytes that had been treated with either HT or ethanol, the control vehicle, for different periods of time by utilizing a peptide as exogenous substrate (8). This assay showed no activation of Akt in the adipocytes after a 1-h stimulation, but after 6 h, there was a 20-fold stimulation (Fig. 2*C*), and the activity remained elevated after 16 h. In contrast, the A2myrAkt Δ4–129-ER construct was not significantly stimulated at any of the times tested, and the myrAkt Δ4–129-ER was not activated in the absence of HT (Fig. 2*C*). The slower rate of activation of myrAkt Δ4–129-ER with HT in the adipocytes could be due to the lower level of expression of these constructs in the adipocytes in comparison to the NIH3T3 cells or for some other unknown reason.

To measure the enzymatic activity of the expressed myrAkt Δ4–129-ER in comparison to the endogenous Akt, 3T3-L1 adipocytes expressing myrAkt Δ4–129-ER were stimulated with HT for 16 h or 10 min with 100 nM insulin and lysed, and the expressed Akt and endogenous Akt were immunoprecipitated with the anti-HA antibody and an antibody to the PH domain of Akt, respectively. The immunoprecipitates were tested for kinase activity *in vitro* using the peptide as substrate (8). The immunoprecipitated endogenous Akt had approximately 5 times as much activity as the expressed myrAkt Δ4–129-ER.

We next evaluated the ability of this conditionally active Akt to regulate glucose transport. The uptake of 2-[^{3}H]deoxyglucose was measured in differentiated adipocytes expressing either myrAkt Δ4–129-ER or A2myrAkt Δ4–129-ER that had been treated for various periods of time with HT. Exposure of cells expressing myrAkt Δ4–129-ER to HT for 1 h or less did not result in any significant change in glucose uptake (Fig. 5*A*), consistent with the low activation of Akt enzymatic activity at these times in adipocytes (Fig. 2*C*). In contrast, the 16-h stimulation was associated with a 2.5–3-fold increase in 2-[^{3}H]deoxyglucose uptake compared with both control-treated cells and cells expressing A2myrAkt Δ4–129-ER that were control-treated or treated with HT (Fig. 5A). Incubations of

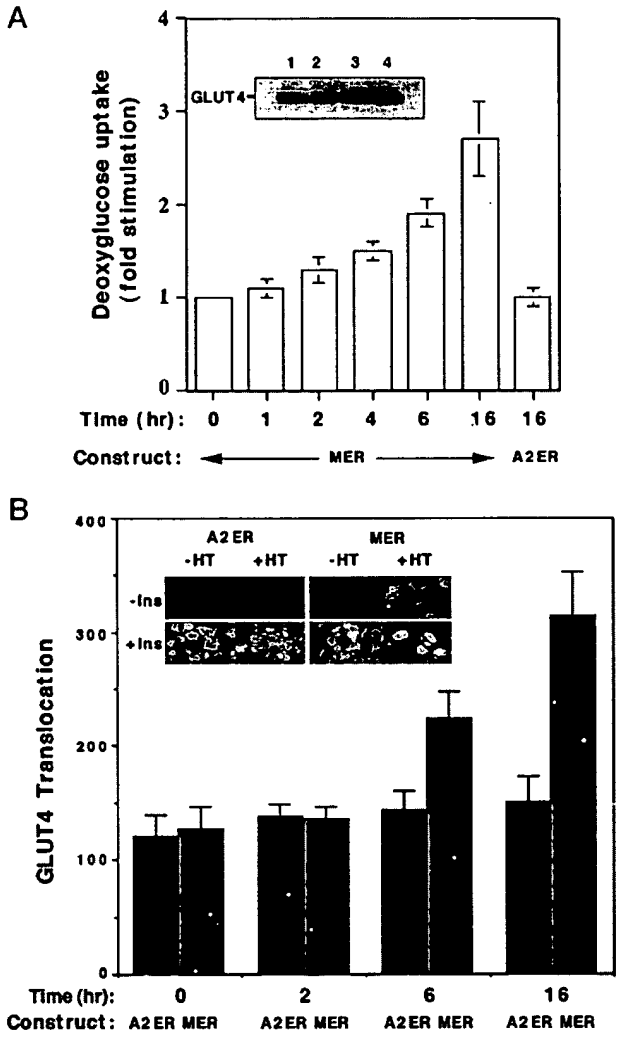

FIG. 5. **Activation of glucose uptake and GLUT4 translocation by myrAkt Δ4–129-ER in 3T3-L1 adipocytes.** *A*, glucose uptake. 3T3-L1 cells expressing either myrAkt Δ4–129-ER (*MER*) or A2myrAkt Δ4–129-ER (*A2ER*) were differentiated into adipocytes. Both cell lines were treated with either 1 μM HT or control vehicle for the indicated times, and then 2-[^{3}H]deoxyglucose uptake was measured, as described under "Experimental Procedures." The nonspecific background measured in cells exposed to cytochalasin B in the absence of insulin was subtracted from all values. The results shown are means ± S.E. (n = 3). Fold stimulation refers to the increase in 2-[^{3}H]deoxyglucose uptake observed in the presence of HT over that in its absence. In the *inset* is shown the GLUT4 levels of MER cells (*lanes 1* and *2*) and A2ER cells (*lanes 3* and *4*) treated for 16 h with HT (*lanes 2* and *4*) or not (*lanes 1* and *3*). *B*, GLUT4 translocation. 3T3-L1 cells expressing either myrAkt Δ4–129-ER (*MER*) or A2myrAkt Δ4–129-ER (*A2ER*) were differentiated into adipocytes and treated or not treated with 1 μM HT for the indicated times, and the amount of surface GLUT4 determined as described under "Experimental Procedures." Results shown are means ± S.E. in arbitrary units in which at least six fields were scored per condition. The *inset* shows a representative field of cells treated or not treated with HT for 16 h.

cells expressing A2myrAkt Δ4–129-ER with HT for shorter periods of time (2–6 h) gave a partial stimulation of glucose uptake. This increase in glucose uptake was not associated with a change in the level of expression of GLUT4, as determined by immunoblotting crude total membrane fractions (Fig. 5*A*, *inset*).

To assess whether activation of this conditionally active Akt could stimulate GLUT4 translocation to the plasma membrane, the sheet assay was utilized (19, 20). Cells expressing either myrAkt Δ4–129-ER or A2myrAkt Δ4–129-ER were treated or not for various periods of time with HT and sonicated, and the amount of plasma membrane GLUT4 was measured. Exposure of cells expressing myrAkt Δ4–129-ER to HT for 2 h or less did not result in any significant change in surface GLUT4 (Fig. 5*B*), consistent with the low activation of Akt enzymatic activity at these times in adipocytes (Fig. 2*C*). After 16 h, HT stimulated a 3-fold increase in the amount of surface GLUT4 in the cells expressing myrAkt Δ4–129-ER but not those expressing A2myrAkt Δ4–129-ER (Fig. 5*B*). After a 6-h stimulation, a smaller increase in surface GLUT4 was also observed in the cells expressing myrAkt Δ4–129-ER. Insulin stimulated GLUT4 translocation approximately 7–8-fold in both cell types (Fig. 5*B*, *inset*). Thus, the conditionally active Akt could regulate 2-[^{3}H]deoxyglucose uptake and GLUT4 translocation in 3T3-L1 adipocytes.

The Conditionally Active Akt Rapidly Stimulates PHAS-I Phosphorylation in NIH3T3 Cells—In addition to regulating glucose uptake, insulin stimulates protein synthesis in a variety of cell types (31). The constitutively active Akt, myrAkt, was found to activate protein synthesis to a level comparable to insulin stimulation.[2] One mechanism whereby insulin has been found to regulate protein synthesis is via the phosphorylation of the eIF4E-binding protein, PHAS-I (31). The nonphosphorylated form of PHAS-I inhibits protein synthesis by tightly binding to the mRNA cap-binding protein, eIF4E. When PHAS-I is phosphorylated on the appropriate sites, it releases eIF4E, which is then free to participate in translation initiation (31). The phosphorylation of PHAS-I has been shown to be controlled by a rapamycin-sensitive pathway involving the mammalian target of rapamycin, mTOR (31). There is also evidence that PI 3-kinase is an upstream element in this pathway, but the role of Akt in the control of PHAS-I phosphorylation is not known.

Experiments were therefore performed to investigate the effect of the conditionally active Akt on the phosphorylation of PHAS-I and its association with eIF4E. NIH3T3 cells expressing myrAkt D4–129-ER (MER-3T3) or control cells were treated with 1 μM HT and lysed, and the lysates were either analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting for PHAS-I or first incubated with m^{7}GTP-Sepharose to determine the amount of PHAS-I complexed with eIF4E. Phosphorylation of PHAS-I has been shown to markedly decrease its electrophoretic mobility in SDS gels (18). By gel shift analysis, HT treatment of MER-Akt-expressing cells was found to rapidly stimulate PHAS-I phosphorylation to an extent comparable to insulin (Fig. 6*A*). In contrast, HT was without effect in the control cells (Fig. 6*A*), whereas insulin stimulated the shift of PHAS-I in both cell types. In addition, tamoxifen stimulated a dissociation of PHAS-I from eIF4E in the MER-Akt cells but not in the control cells, as detected by a decrease in the amount of the PHAS-I/eIF4E complex bound to the m^{7}GTP-Sepharose (Fig. 6).

DISCUSSION

The results presented here demonstrate that fusion of the hormone binding domain of the estrogen receptor to an activated form of Akt renders the kinase activity of this protein dependent on the addition of exogenous HT. Using HT, the kinase activity can be rapidly turned on in several cell types, including NIH3T3 cells (Fig. 2*A*), a mouse hepatoma cell line, and primary myoblasts.[3] The induction of Akt kinase activity correlates with the phosphorylation of this protein, including at a key regulatory site, Ser^{473} (Fig. 2, *B*, *D*, and *E*), consistent

[2] A. D. Kohn and R. A. Roth, unpublished observations.
[3] Andreas Barthel and R. A. Roth, unpublished observations.

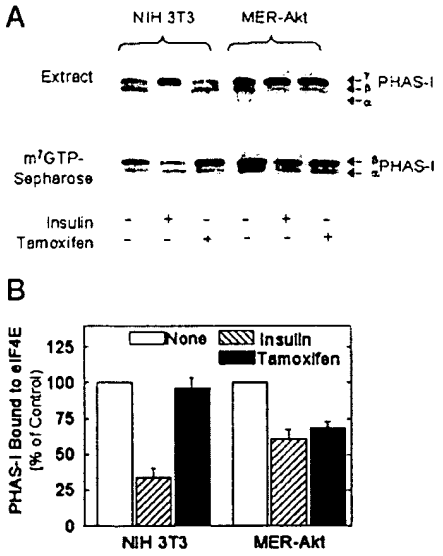

FIG. 6. **PHAS-I shift and dissociation from eIF4E induced by activation of myrAkt Δ4–129-ER.** NIH3T3 cells expressing either myrAkt Δ4–129-ER (*MER-Akt*) or not (*NIH3T3*) were treated with either 1 μM HT (tamoxifen) or insulin for 30 min or control vehicle as indicated and lysed; the total lysates were either analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting for PHAS-I (*upper blot*; extract) or incubated with m7GTP-Sepharose; and the bound material was eluted and blotted for PHAS-I. The blots from a representative experiment are shown in *A*, and the means ± S.E. from three experiments are shown in *B*.

with the hypothesis that phosphorylation regulates the enzymatic activity of this chimera as it does the the wild type enzyme (32). The rapid activation of the chimera with HT treatment is consistent with the previously described model, in which hormone binding stimulates the release of the 90-kDa heat shock protein from the HBD, thereby allowing access of other proteins (*e.g.* a kinase) to the chimeric molecule (33). An alternative model would be that the HBD could induce oligomerization of the chimera, and this oligomerization could be sufficient to result in transphosphorylation and activation of Akt. This did not appear to be the case because A2myrAkt Δ4–129-ER, which only lacks the glycine that is myristoylated, was not activated. Thus, the Akt chimera must be present in a membrane compartment to be activated. Presumably, this is due to the presence of a kinase in the membrane that is responsible for phosphorylating Akt. This kinase could be the recently described Akt kinase kinase, which is stimulated by PI 3,4,5-trisphosphate and PI 3,4-bisphosphate (7). Alternatively, a distinct membrane kinase could also be involved. In this regard, it is intriguing that wortmannin and LY294002, inhibitors of PI 3-kinase, could block the activation of myrAkt Δ4–129-ER by HT (Fig. 3 and data not shown). The latter has recently been shown to also cause a deactivation of the constitutively active Akt (34). This inhibition could be caused by these drugs directly inhibiting the kinase that is responsible for phosphorylating Akt (possibly the PI 3-kinase itself) or, more likely, by the ability of these drugs to lower the basal levels of PI 3-phosphates in the cells and thereby decrease the basal activity of a kinase sensitive to these compounds. The slower rate of activation of myrAkt Δ4–129-ER in 3T3-L1 adipocytes in comparison to the NIH3T3 cells could be due to a lower level of the membrane kinase responsible for phosphorylating and activating Akt and/or a lower basal level of the necessary PI 3-phosphates. Alternatively, it may be due to the lower levels of expression of the Akt chimera in these cells or for some other reason.

The generation of a conditionally active Akt allowed us to investigate downstream targets of this enzyme. Prior studies have shown that expression of different constitutively active forms of Akt induce various subsequent biological responses, including stimulation of the p70 S6-kinase and glucose uptake (4, 8–13). In the present study, we could show activation of the p70 S6-kinase activity in NIH3T3 cells expressing myrAkt Δ4–129-ER after only a 10-min stimulation with HT (Fig. 4), a time very close to that required for induction of Akt enzymatic activity in these cells (Fig. 2*A*). These results indicate that the activation of the 70-kDa S6 kinase by Akt is an immediate consequence of stimulation of Akt kinase activity, rather than an event secondary to long-term expression of a constitutively active kinase.

In 3T3-L1 adipocytes, the activation of myrAkt Δ4–129-ER was considerably slower than in NIH3T3 cells (Fig. 2*C*), and the maximal amount of activity was less. A detectable increase in glucose uptake was observed after 4- and 6-h incubations with HT. After 16 h, glucose uptake was stimulated approximately ¼ as well as a maximal dose of insulin. The extent of stimulation of glucose uptake after HT treatment corresponded with the extent of GLUT4 translocation and the relative amounts of Akt stimulated by HT in comparison to insulin. That is, the amount of Akt kinase activity and glucose uptake observed with a 16-h HT stimulation of cells expressing myrAkt Δ4–129-ER was approximately ¼ the maximal amount of glucose uptake and endogenous Akt kinase activity observed in insulin-treated cells. It is possible that the longer period of time required to maximally stimulate Akt activity and glucose uptake in these cells allowed other processes to occur in addition to Akt activation, raising the question of whether Akt activation alone is sufficient for stimulation of glucose uptake. For example, it was possible that Akt activation stimulates the release of a secreted molecule that feeds back to activate glucose uptake. However, the supernatants of these cells were incapable of stimulating glucose uptake in 3T3-L1 adipocytes,[3] indicating that this increase in glucose uptake was at least not due to the release of a secreted factor. Recent additional evidence of a role for Akt in mediating the insulin-induced increase in glucose uptake has come from studies showing that expression of an inactive Akt can hinder the ability of insulin to stimulate GLUT4 translocation in adipocytes (35).

In the present study, we also utilized the conditionally active Akt to determine whether this enzyme can modulate the phosphorylation of PHAS-I, a key regulator of protein synthesis, as well as its association with eIF4E (31). A constitutively active Akt, myrAkt, can increase protein synthesis in 3T3-L1 adipocytes to the same extent as insulin[2] and stimulate an increase in leptin production in these cells (36). One mechanism by which insulin and other growth factors regulate protein synthesis is a stimulation in phosphorylation of PHAS-I and its subsequent release from eIF4E (31). Prior studies have demonstrated that insulin stimulates a phosphorylation of PHAS-I and its subsequent release from eIF4E, thereby increasing the amount of eIF4E available to participate in translation of capped mRNAs (31). In the present study, we showed that activation of the conditionally active Akt caused a shift of the PHAS-I protein within 10 min of stimulation (Fig. 6). This phosphorylation was associated with a release of PHAS-I from eIF4E. Thus, acute activation of Akt was sufficient to mimic the ability of insulin to stimulate PHAS-I phosphorylation and release of eIF4E, indicating that Akt is upstream of the kinase(s) responsible for phosphorylation of PHAS-I.

In summary, the present study provides further evidence that Akt can directly regulate activation of the 70-kDa S6 kinase and glucose uptake. In addition, we provide the first

evidence that activation of Akt can regulate the phosphorylation of PHAS-I, as well as its association with eIF4E, providing a potential mechanism whereby insulin may regulate protein synthesis.

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Commentary

AKT plays a central role in tumorigenesis

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AKT is emerging as a central player in tumorigenesis. In this issue of PNAS, Mayo and Donner (1) report on yet another function of AKT, involving regulation of the Mdm2/p53 pathway.

The first evidence pointing to a role of AKT in oncogenesis was provided by early studies of transforming viruses. A novel retrovirus, isolated from an AKR mouse T cell lymphoma (2), harbored transduced sequences of cellular origin (3). In 1991, our collaborative studies with Philip Tsichlis and Stephen Staal resulted in the cloning of the viral oncogene v-*akt* (4). The predicted oncoprotein contained viral Gag sequences fused to a kinase related to protein kinase C. The oncogenic potential of v-akt arises from the creation of a myristylation site at the amino terminus and consequent constitutive kinase activity (5). By different approaches, aimed at identifying novel protein kinases, two other groups independently cloned the identical cellular sequence at about the same time (6, 7). *AKT* is now known to define a family of closely related, highly conserved cellular homologues (reviewed in ref. 8). In human, these are designated *AKT1*, *AKT2*, and *AKT3*, located at chromosomes 14q32, 19q13, and 1q44, respectively (reviewed in ref. 9). The encoded proteins are serine/threonine kinases belonging to the protein kinase B (PKB) family, and the AKT1, AKT2, and AKT3 proteins are also known as PKBα, PKBβ, and PKBγ, respectively. Each AKT family member contains an amino-terminal pleckstrin homology (PH) domain, a short α-helical linker, and a carboxyl-terminal kinase domain (8). PH domains exist in diverse signaling molecules and permit anchorage of proteins to the cell membrane via phospholipid interactions (10).

The degree of functional redundancy between AKT1, AKT2, and AKT3 is currently unclear. Although each kinase responds similarly to various stimuli, their different tissue-specific expression patterns suggest distinct roles, e.g., compared to *Akt1*, *Akt2* transcripts are especially abundant in highly insulin-responsive tissues such as brown fat (11). Moreover, *Akt2* knockout mice exhibit impaired ability of insulin to lower blood glucose as a result of defects in the action of the hormone on liver and skeletal muscle (12). Expression of Akt1 and Akt3 does not compensate for loss of Akt2, thus establishing *Akt2* as an essential gene for the maintenance of normal glucose homeostasis.

Mounting evidence suggests that AKT perturbations play an important role in human malignancy. In 1992, we reported the first recurrent involvement of an AKT gene in a human cancer, demonstrating amplification and overexpression of *AKT2* in ovarian tumors and cell lines (13). Subsequent studies documented *AKT2* amplification and/or mRNA overexpression in 10–20% of human ovarian and pancreatic cancers (14, 15) and activation of the AKT2 kinase in ≈40% of ovarian cancers (16). Overexpression of AKT2 can transform NIH 3T3 cells (17), and *AKT2* antisense RNA inhibits the tumorigenic phenotype of cancer cells exhibiting amplified *AKT2* (15). Amplification of *AKT1* was observed in a human gastric cancer (3), and AKT1 kinase activity is often increased in prostate and breast cancers and is associated with a poor prognosis (18). To date, amplification of *AKT3* has not been described. However, *AKT3* mRNA is up-regulated in estrogen receptor-negative breast tumors, and increased AKT3 enzymatic activity was found in estrogen receptor-deficient breast cancer and androgen-insensitive prostate cancer cell lines (19), suggesting that AKT3 may contribute to the aggressiveness of steroid hormone-insensitive cancers.

There has been enormous interest in the mechanisms and cellular consequences of signal propagation from receptor tyrosine kinases to AKT (reviewed in refs. 8 and 20–28). The AKT kinases are major downstream targets of growth factor receptor tyrosine kinases that signal via phosphatidylinositol 3-kinase (PI3K).

AKT activation is a multistep process involving both membrane translocation and phosphorylation (29). The pleckstrin homology domain of AKT kinases has affinity for the 3′-phosphorylated phosphoinositides 3,4,5-trisphosphate (PI-3,4,5-P_3) and PI-3,4,-P_2 produced by PI3K, and they are activated specifically by the latter lipid. Phospholipid binding triggers the translocation of AKT kinases to the plasma membrane. Upon membrane localization, AKT molecules are phosphorylated at Thr-308/309 in the kinase activation loop and Ser-473/474 in the carboxyl-terminal tail. Thr-308/309 phosphorylation is necessary for AKT activation, and Ser-473/474 phosphorylation is only required for maximal activity. Phosphorylation on these residues is induced by growth factor stimulation and inhibited by the PI3K inhibitor, LY294002. Indeed, the kinase responsible for Thr-308/309 phosphorylation, PDK1 (for 3-phosphoinositide-dependent kinase) is activated by the PI3K lipid products PI-3,4,5-P_3 and PI-3,4-P_2. More controversial is the identity of PDK2, the kinase(s) responsible for Ser-473/474 phosphorylation (30). Interestingly, avian sarcoma virus 16 contains a potent transforming sequence derived from the cellular gene for the catalytic subunit of PI3K (31), and its human homologue, *PIK3CA*, was implicated as an oncogene in human ovarian cancer (32). Furthermore, the negative regulator of this pathway, the tumor suppressor PTEN, inhibits AKT activation by dephosphorylating PI-3,4,-P_2/PI-3,4,5-P_3 (reviewed in refs. 33 and 34).

Recent studies have revealed a burgeoning list of AKT substrates implicated in oncogenesis (reviewed in ref. 26). Among its pleiotropic effects, activated AKT is a well-established survival factor, exerting anti-apoptotic activity by preventing release of cytochrome *c* from mitochondria and inactivating forkhead transcription factors known to induce expression of pro-apoptotic factors such as Fas ligand. AKT phosphorylates and inactivates the pro-apoptotic factors BAD and pro-caspase-9. Moreover, AKT activates IκB kinase, a positive regulator of NF-κB, which results in transcription of anti-apoptotic genes. AKT kinases also phosphorylate and inactivate glycogen synthase kinase 3, thereby stimulating glycogen synthesis (35). AKT activation affects cell cycle progression, through regulation of cyclin D stability (36) and inhibition of $p27^{Kip1}$ protein levels (37), and mRNA translation, via phosphorylation of 4E-BP1 and its dissociation from the mRNA cap binding protein eIF4E

See companion article on page 11598.

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(38). Furthermore, AKT mediates the activation of endothelial nitric oxide synthase, an important modulator of angiogenesis and vascular tone (39, 40). Germane to this, infection of the chicken wing web with RCAS retroviral vector expressing activated forms of mammalian Akt leads to the formation of hemangiosarcomas, malignant tumors of vascular cells (41). AKT activation also enhances telomerase activity via phosphorylation of the human telomerase reverse transcriptase subunit (42).

Until recently, it has been difficult to identify unifying themes in AKT substrates. Now, the study by Mayo and Donner (1) and other recent work (ref. 43 and G. Viglietto, personal communication) define an emerging mechanism driven by AKT phosphorylation, namely regulation of nucleo-cytoplasmic localization of critical substrates involved in cell cycling and apoptosis. Mayo and Donner show that phosphorylation by AKT is necessary for nuclear translocation of Mdm2. The oncoprotein Mdm2 and the tumor suppressor p53 are part of an autoregulatory feedback: *Mdm2* transcription is induced by p53, and the Mdm2 protein, in turn, binds the p53 transactivation domain, inhibiting expression of p53-regulated genes involved in cell cycle arrest and apoptosis. In the absence of the p19/p14^{ARF} tumor suppressor, the Mdm2-p53 complex shuttles from the nucleus to the cytoplasm where p53 is targeted for ubiquitin (Ub)/proteasome-mediated degradation (reviewed in ref. 44). Mayo and Donner demonstrate that in serum-starved cells, Mdm2 localizes in the cytoplasm in a complex with AKT. After growth factor stimulation, Mdm2 is phosphorylated by AKT, rapidly dissociates from the complex and enters the nucleus; this leads to reduction of both p53 levels and transactivation (Fig. 1). This study establishes a novel mitogen-regulated pathway linking PI3K/AKT and Mdm2/p53. However, this pathway only regulates nuclear entry of Mdm2, and additional components, e.g., relative levels of p19/p14^{ARF}, are required for a full effect on p53-dependent cell cycle arrest/apoptosis.

Whereas AKT acts in concert with the oncoprotein Mdm2, a recent study by Zhou *et al.* (43) indicates that AKT restrains the tumor suppressor p21^{WAF1}. In breast cancer cells exhibiting AKT activation due to HER-2/neu overexpression, phosphorylation by AKT prevents nuclear localization of p21^{WAF1}, separating this cell cycle inhibitor from its cyclin/cyclin-dependent kinase targets (Fig. 1). Thus, AKT activation antagonizes p21^{WAF1}-mediated cell cycle arrest (43). Cytoplasmic p21 binds to the apoptosis-signal-regulating kinase (ASK1), inhibiting apoptosis. Similarly, recent work revealed

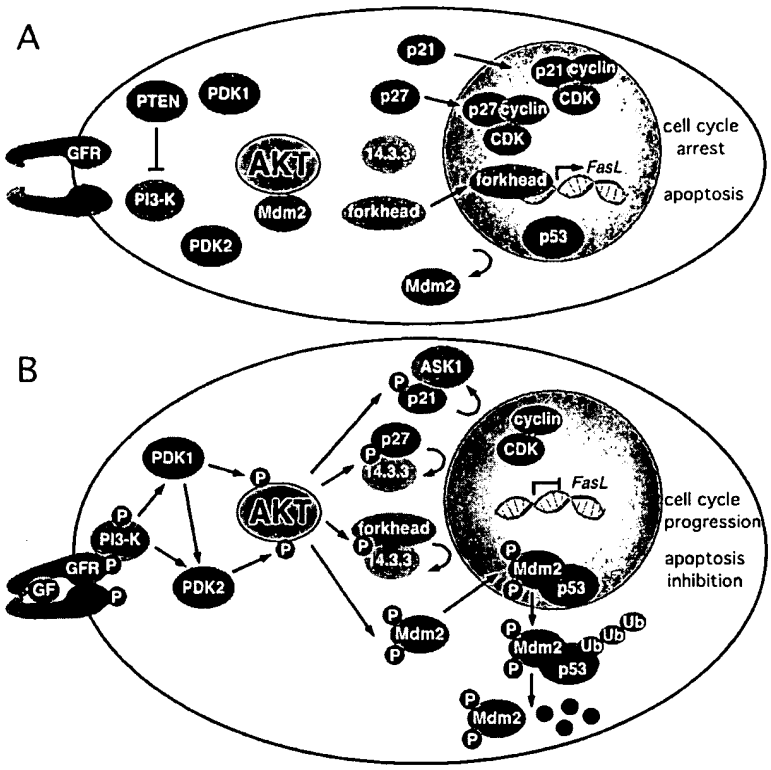

Fig. 1. Phosphorylation by AKT regulates compartmentalization of multiple substrates involved in cell cycle progression and inhibition of apoptosis. (*A*) In serum-starved cells, the pro-apoptotic transcription factors of the forkhead family and cell cycle inhibitors p21 and p27 localize in the nucleus, whereas the oncoprotein Mdm2 is restrained in the cytoplasm. (*B*) After growth factor (GF) stimulation and phosphorylation by AKT, the subcellular localization of these AKT substrates is diametrically changed, contributing to cell cycle progression and inhibition of apoptosis. Cytoplasmic p21 can bind to the apoptosis signal-regulating kinase (ASK1), inhibiting apoptosis. In the absence of p19/p14^{ARF} induction, the Mdm2-p53 complex shuttles into the cytoplasm where p53 is ubiquitinated (Ub) and targeted for degradation.

that phosphorylation of p27^{Kip1} by AKT results in cytoplasmic retention of this cell cycle inhibitor and loss of its growth inhibition (G. Viglietto, personal communication). Cytoplasmic retention of AKT-phosphorylated p27^{Kip1} occurs, at least partly, by binding to the 14.3.3 scaffold protein (Fig. 1). Interestingly, binding to 14.3.3 had been previously reported for the forkhead family transcription factor FKHRL1 after AKT phosphorylation (45), which again is associated with cytoplasmic sequestration of the substrate (Fig. 1). In all these cases, regulation of substrate compartmentalization by AKT appears to be a consequence of phosphorylation near nuclear localization/nuclear export sequences, presumably affecting their net charge and/or conformation. In some cases, binding of the AKT-phosphorylated substrate to 14.3.3 may also affect subcellular localization.

As proposed by Hanahan and Weinberg (46), most tumor-related genetic/epigenetic changes are representative of a finite set of physiological alterations that collectively drive a cell toward malignancy. Based on the evidence outlined above and in Table 1, AKT signaling appears to play a prominent role in several processes considered hallmarks of cancer. Growth signal autonomy would not appear to be a direct effect of AKT signaling. However, overexpression of AKT may permit a tumor cell to become overly responsive to ambient levels of growth factors that normally would not provoke proliferation. Moreover, AKT activation can upregulate insulin-like growth factor I receptor expression (47), and overexpression of growth factor receptors may facilitate oncogenic signaling (reviewed in ref. 46). AKT activation may contribute to tumor invasion/metastasis by stimulating secretion of matrix metalloproteinases (48).

The involvement of AKT in diverse tumorigenic activities suggests that AKT activation alone might be sufficient to induce cancer. However, whereas overexpression of myristylated forms of Akt1, Akt2, and Akt3 are strongly oncogenic, wild-type forms of Akt are only poorly

Table 1. Hallmarks of cancer and the multiple roles of AKT

Cancer hallmarks (46)	Akt functions/substrates (in bold)
Acquired growth signal autonomy	Overexpression of AKT may mediate hyper-responsiveness to ambient levels of growth factors
Insensitivity to antigrowth signals	Promotes nuclear entry of **Mdm2**, thus inhibiting p53 pathway
	Induces cytoplasmic localization of **$p21^{WAF1}$** and **$p27^{Kip1}$**, promoting cell growth
	Stabilizes cyclin D1/D3
Inhibition of programmed cell death	Inactivates pro-apoptotic factors **BAD** and **(pro)caspase-9**
	Activates **IKK**, resulting in NF-κB transcription of anti-apoptotic genes
	Inactivates **forkhead transcription factors**, thereby inhibiting expression of Fas ligand
Unlimited replicative potential	Enhances telomerase activity by phosphorylation of **hTERT**
Sustained angiogenesis	Activates **eNOS**, thus promoting angiogenesis
Tissue invasion and metastasis	Contributes to invasiveness by stimulating secretion of MMP

IKK, IκB kinase; hTERT, human telomerase reverse transcriptase; eNOS, endothelial nitric oxide synthase; MMP, matrix metalloproteinase.

transforming (5, 41). Nevertheless, it is noteworthy that in human chronic myeloid leukemia a single chromosome change, leading to the creation of the BCR/ABL oncogenic tyrosine kinase, is considered sufficient to transform bone marrow cells, and activation of the PI3K/AKT pathway is essential for this process (49). Interestingly, in an experimental setting the oncogenic effects of Akt1, Akt2, and Akt3 were indistinguishable (41), suggesting that the downstream targets relevant to oncogenic transformation may be shared by the three AKT kinases. Whether the various AKT isoforms have some distinguishing substrates in human malignancy or have different, functionally pertinent binding affinities for other interacting proteins, such as the adaptor APPL (50), remains to be determined. Clearly, the expanding number of substrates implicated in various aspects of tumorigenesis highlights the central role of AKT kinases in many human cancers. For this reason, we anticipate that much attention will be given to the identification of inhibitors or modulators of the PI3K/AKT pathway, with the intention of developing novel therapeutic strategies directed at neoplasms exhibiting AKT activation.

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Brief report

The AKT kinase is activated in multiple myeloma tumor cells

Jung-hsin Hsu, Yijiang Shi, Stanislaw Krajewski, Stephen Renner, Myrna Fisher, John C. Reed, Thomas F. Franke, and Alan Lichtenstein

Immunohistochemistry (IHC) was performed on archived bone marrow (BM) with a phosphospecific anti-AKT antibody. IHC on 26 BM biopsies from patients with multiple myeloma (MM) demonstrated phospho-AKT staining of malignant plasma cells in a cell membrane–specific pattern, whereas nonmalignant hematopoietic cells did not stain. Preabsorption of the antibody with phosphorylated AKT peptide, but not nonphosphorylated peptide, abrogated staining. Frequency of plasma cell staining in BMs of patients with stage I or smoldering MM was significantly less than that of stage III MM marrows. Plasma cells in 10 patients with monoclonal gammopathy of undetermined significance were not stained by the antibody. To investigate the significance of AKT activation, 2 cell lines initiated from cultures of primary MM cells were also studied. Both demonstrated constitutive AKT activation. Interruption of AKT activation and activity, achieved by either exposure to wortmannin or by ectopic expression of a dominant negative AKT mutant, resulted in inhibition of MM cell growth in vitro. These results indicate that activation of the AKT kinase is a characteristic of MM cells and suggest that AKT activity is important for MM cell expansion. (Blood. 2001;98:2853-2855)

Introduction

In multiple myeloma (MM), enhanced proliferation and resistance to apoptosis account for expansion of the malignant clone.[1,2] Previous investigations have demonstrated alteration of the ERK,[3] jun kinase,[4] STAT,[5] and AKT kinase[6] signaling cascades in MM cells and implicated the pathways in clonal expansion. These studies, however, were mainly investigations in cell lines, and it is unclear whether the observations can be extrapolated to tumors in patients. We therefore used immunohistochemical (IHC) staining to test activation of AKT in situ in MM cells of patients. AKT is activated downstream of phosphatidylinositol 3-kinase (PI3-kinase) by translocation to the cell membrane[7] and phosphorylation at Ser and Thr residues.[8] Its activity is inhibited by the tumor suppressor PTEN phosphatase. Our IHC assay with a phosphospecific anti-AKT antibody confirmed frequent AKT activation in MM cells of patients with stage III disease. Further evaluation of 2 MM cell cultures, recently explanted from patients, indicated that AKT activation may play a role in MM cell expansion.

Study design

Myeloma cells

Bone marrow (BM) cells from one MM patient and peripheral blood from a second with plasma cell leukemia were separated by an immunoabsorption column to isolate high CD38-expressing cells (as described by Tu et al[6]). The separated cells consisted of more than 98% plasma cells by light microscopy. These cells were cultured in vitro without growth factors in complete RPMI media. After 1 week, the cultures were composed of 100% malignant plasma cells that began to slowly proliferate and have been maintained in culture for at least 4 months. These cells morphologically resemble plasma cells and express high levels of membrane CD38 and monoclonal cytoplasmic light chain. Their doubling time is approximately 60 hours.

Transient transfections and flow cytometry

The kinase-inactive AKT construct, HA-AKT(K179M), functions as a dominant negative inhibitor of endogenous AKT.[9] It was cloned into the enhanced green fluorescence protein (EGFP)–expressing vector, pEGFP-C2 (Clontech) at *Hin*dIII/*Sma*I sites. Myeloma cells were transiently transfected with HA-AKT(K179M) or empty pEGFP-C2 vector by electroporation (250 V for 25 ms). At 24 hours after transfection, viable cells were stained with the DNA dye Hoechst at 2.5 μg/mL for 20 minutes and cell cycle distribution was determined by first gating on EGFP-fluorescing cells. EGFP fluorescence demonstrated 8% to 10% transfection efficiency for both HA-AKT(K179M) and empty vector.

Patient population

Archival BM biopsies obtained from patients at Kaiser Permanente Hospital (Woodland Hills, CA) were immunostained for phosphorylated AKT. Biopsies were obtained at the time of diagnosis between 1990 and 1999 and clinically staged by the Durie-Salmon system. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering MM were diagnosed in patients as previously described.[10,11]

IHC staining

Immunohistochemical staining was performed on Bouin-fixed biopsies as previously reported,[12] using a diaminobenzadine-based detection method. The phosphospecific anti-AKT antibody, obtained from Bioscience (catalog no. 44-622; Princeton, NJ), which recognizes phosphorylated AKT, was used as the primary antibody. Negative controls, where the primary anti-AKT antibody was

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omitted, were uniformly negative for immunostaining. In preabsorption studies, the phosphospecific anti-AKT antibody was preadsorbed with 1 to 2 μg/mL of either phosphorylated AKT peptide (sequence = Ac-C(Ahx)KHFPQF(pS)YSAS-NH_2 or nonphosphorylated peptide (sequence = Ac-C(Ahx)KHFPQFSYSAS-NH_2). (Single-letter amino acid codes used.) Frequency of phospho-AKT expression was determined by counting at least 250 plasma cells from 3 different areas in BMs from patients with myeloma. For patients with MGUS, only 50 to 100 plasma cells were enumerated.

Western blot analysis

Western blot analysis was performed as previously described.[13] Densitometric analysis was used to determine the median effective dose (ED_{50}) for wortmannin (drug dose inhibiting phosphorylation by 50%).

Statistics

The *t* test was used to determine significance.

Results and discussion

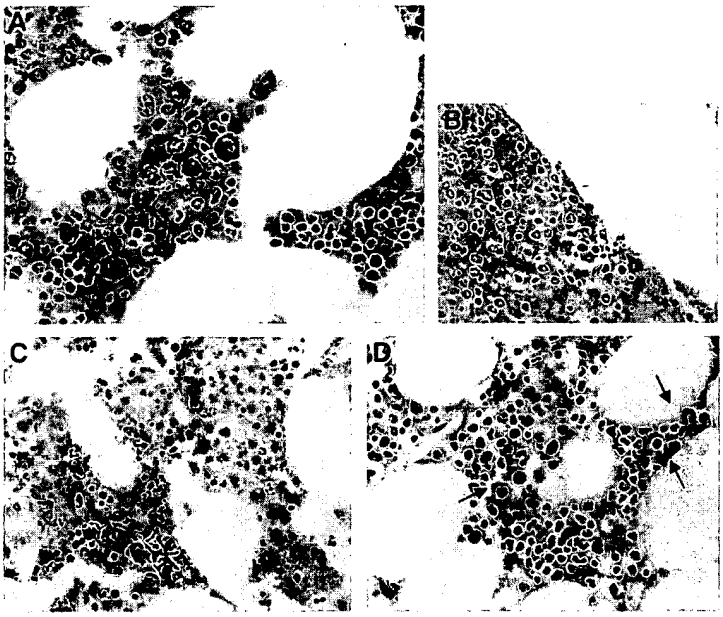
AKT activation in myeloma BM biopsies

The AKT kinase is activated by phosphorylation at Ser473. We thus tested activation of AKT in myeloma marrow by IHC using a phosphospecific anti-AKT antibody that only detects AKT when it is phosphorylated at Ser473. As shown in Figure 1, the phospho-AKT antibody readily stained myeloma plasma cells. Immunostaining was present in a cell membrane–specific pattern, consistent with the known subcellular locale when AKT becomes activated. Of note, staining was specific for myeloma tumor cells because nonmalignant hematopoietic cells in these same biopsies were negative. In 3 of the BM samples, immunostaining of MM cells was abrogated when the antibody was absorbed with phosphorylated, but not nonphosphorylated, AKT peptide, attesting to the specificity of immunostaining. Examples of peptide preabsorption in one BM sample are shown in panels B and C of Figure 1.

The percent of malignant plasma cells positively stained in these biopsies was significantly ($P < .05$) higher in Durie-Salmon stage III patients than in stage I (Figure 1E). Frequency of staining in stage I disease was comparable to that of indolent MM. No plasma cell immunostaining was detected in marrows from 10 patients with MGUS (Figure 1E). Although the degree of plasma cell infiltration of these MGUS marrows was low (mean 2%), the plasma cells were easily identified and were clearly nonreactive with the antibody (Figure 1D, arrows).

Inhibition of AKT activation inhibits MM cell growth

We next studied MM cell populations from 2 patients; the cells were successfully cultured over a 4-month period. Immunoblotting demonstrated constitutive phosphorylation of AKT which, in one case (UCLA no. 1), was sensitive to the PI3-kinase inhibitor, wortmannin (Figure 2A). Wortmannin also induced a cytoreductive



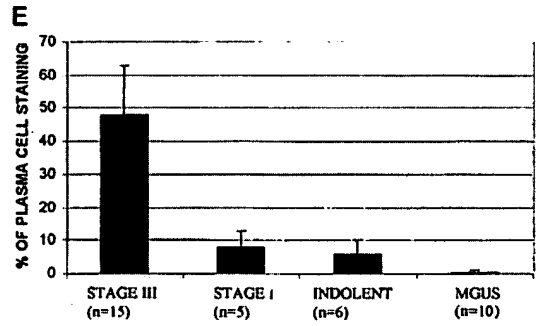


Figure 1. Immunohistochemistry of BM stained with phosphospecific anti-AKT antibody. (A) Myeloma marrow with immunostained plasma cells in a membrane-specific pattern. (B) Myeloma marrow stained with antibody after antibody was first preabsorbed with phosphorylated AKT peptide. (C) Myeloma marrow (same as used in panel B) stained with the same antibody after antibody was preabsorbed with nonphosphorylated AKT peptide. (D) MGUS marrow stained with phosphospecific AKT antibody. Three plasma cells are shown with arrows; original magnification × 400. (E) Frequency (mean ± SD) of plasma cells immunostaining in patient marrows.

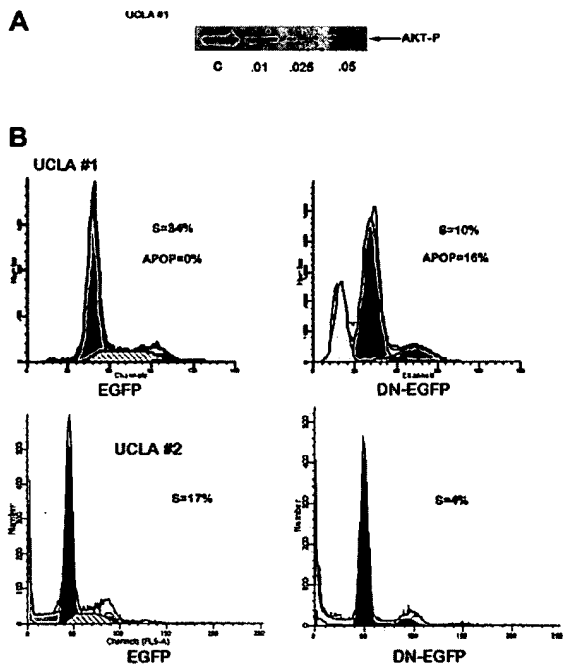


Figure 2. Inhibition of AKT activity in MM cells curtails cell growth and S-phase distribution. (A) UCLA no. 1 cells were treated with increasing concentrations of wortmannin (shown below protein bands in μM) for 2 hours and Western blot then performed with phosphospecific AKT antibody. Immunoblot for total AKT (not shown) showed no differences in expression of total AKT in all groups. Additional cells treated identically were cultured for 72 hours with the same concentrations of wortmannin and then viable cell recovery was recorded. Mean results of 4 independent experiments were used to determine LD_{50} as described. (B) UCLA no. 1 MM cells (top panels) and UCLA no. 2 cells (bottom panels) were transiently transfected with control EGFP vector (left panels) or EGFP vector expressing DN AKT (DN-EGFP, right panels). Twenty-four hours after transfection, cells were stained with Hoechst dye and cell cycle analysis performed by gating on $EGFP^+$ cells. Percent of cells in S phase or apoptotic (APOP) is shown.

effect on these MM cells and this correlated with its ability to inhibit AKT phosphorylation. The concentration required for 50% inhibition of cell recovery (LD_{50}) after 72 hours was 0.02 μM for UCLA no. 1 cells, whereas the ED_{50} for inhibition of AKT activation was 0.01 μM. Constitutive AKT phosphorylation in the UCLA no. 2 cell line was more resistant to wortmannin with an inhibition detected only at wortmannin concentrations more than 0.1 μM. We also transiently transfected these MM cells with a plasmid expressing a dominant negative *AKT* gene (K179M) fused to the *EGFP* gene, or (as a control) the empty vector expressing only EGFP, and then performed flow cytometry for cell cycle analysis (Hoechst staining) on EGFP-gated cells. The expression of EGFP from both plasmids in both MM cell populations was comparable (7%-10%) after transfection. As shown in representative experiments (Figure 2B), expression of the dominant negative K179M in UCLA no. 1 MM cells resulted in a decreased number of cells in S phase (10% versus 34% in control cells) and increase in apoptosis (16% versus 0%) as shown by a sub-G_1 peak. In UCLA no. 2 cells, expression of the dominant negative AKT resulted in a decrease in S-phase distribution (4% versus 17%). Cell cycle distribution in cells transfected with the control EGFP plasmid were not altered compared to nontransfected cells (not shown). The 2 experiments shown in Figure 2B were repeated twice with identical results.

In this study, AKT was frequently activated in MM cells and the frequency of activation correlated with disease activity, being significantly greater in stage III disease compared to stage I or indolent MM and being undetected in MGUS. We[6] and others[14] have shown that interleukin 6 and insulinlike growth factor 1 can activate AKT in myeloma cells. Thus, the detected AKT activation in MM marrow may have been due to cytokine stimulation in situ. However, as shown in 2 MM cell populations in vitro, constitutive activation may exist. This could be due to autocrine cytokine stimulation, loss-of-function PTEN mutations, or to gain-of-function mutations in AKT or PI3-kinase. Hyun et al[14] found PTEN mutations in some human myeloma cell lines. Thus, PTEN mutations may occur in patients with myeloma and could explain heightened AKT activation.

When the AKT pathway was paralyzed by transient transfection with a dominant negative AKT construct, a decrease in S-phase distribution (and increase in apoptosis in one case) was found in Hoechst-stained cells. This is consistent with the work of Hyun and coworkers[14] who described an inhibitory effect of PTEN transfection in MM cells concurrent with a decrease in AKT activation.

In summary, these results indicate that activation of AKT occurs in MM plasma cells. Because of its central location, activating diverse downstream proliferative and antiapoptotic pathways, AKT is a promising target for future molecular-based therapy. In addition, the frequent activation in myeloma tumor cells compared to nonmalignant cells suggests a therapeutic window may exist in patients.

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